# THE CYSTINE PLUS CYSTEINE AND METHIONINE CONTENTS OF THE SEED PROTEINS, AND SULPHUR DISTRIBUTIONS OF SEEDS OF VICIA SPP. (VETCHES), WITH NOTES ON THEIR CYANOGENESIS

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Apart from some earlier work (e.g., Osborne and Heyl, 1908; Schulze, 1893; and May and Rose, 1922), no adequate data on the proteins and amino acids in Vicia are available. It has been pointed out by Glynn, Himsworth and Neuberger (1945) and others, that the sulphur-containing amino acids may represent a limiting factor in the nutritional value of a diet. Since there are no reports on the contents of these amino acids in Vicia seeds, so far as is known, this study was undertaken to investigate this question. The sulphur distributions and cyanogenesis have been examined in seeds of some representatives of this genus.

The value of such data is emphasized by the recent work of Bailey (1948) which indicates that non-toxic varieties of Vicia may become important in either partially or wholly replacing field peas in parts of Western Australia, both as a dry fodder for the summer period and as a grain legume crop. Varieties so far tested by this worker have, in contrast to field peas, been found either entirely immune to pea-weevil attack or only very slightly affected (<1 p.c.). Some varieties have been found to be resistant to the red-legged earth mite (*Halotydeus destructor*, Tucker) and to black stem rot.

## EXPERIMENTAL.

*The Preparations.*

The Vetch seeds selected for the S-distribution study were of the varieties 11 Vicia sp. W150[2] (commonly known as Vetch) and 191 Vicia dasycarpa Ten. C.P.I. 9,189[2] (common name Auburn Woolly Pod Vetch), grown at Muresk, Western Australia, and harvested ripe, on December 10, 1946 and December 16, 1946 respectively.

The seeds were ground to pass through 1·0 mm. sq. sieve. From the ground seeds "whole" protein preparations[3] were made by extracting non-coagulable and other non-

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1 For the purpose of concise notation and expediency, seeds of Vicia samples are given the distinguishing numbers which will be used throughout this paper. See also Table 5.

2 The symbols such as W150, C.P.I. 9,189 and others, see Table 5, are not meant to be parts of generic names, but are used for recording purposes only, and were supplied by Bailey (1948).

3 "Whole" protein signifies here the total content of all proteins present in the seeds.

protein nitrogenous substances, as well as certain other substances, using the procedure of Lugg (1939a) as modified by Lugg and Weller (1944). Although this procedure retains such impurities as cellulose and some carbohydrates in "whole" protein preparations, it ensures the complete retention of proteins by denaturing and precipitating them, and avoids the damaging of amino acid residues while removing non-protein nitrogenous and S-containing substances. In particular, possible loss of cystine through conversion of its residues into thio-ethers is avoided. (See Horn, Jones and Ringel, 1942). The importance of careful consideration of the methods of preparing or purifying the starting material is discussed by Johanson (1948b) for the specific case of cystine preparation from wool protein.

TABLE 1.

*Preparation of "whole" protein samples from seeds.*

| No. | Seed material. | Fresh wt. taken gm. | P.c. moisture. | Dry wt. gm. | P.c. N. | Total N mg. | Coagulable N mg. | Protein N mg. | Dry wt. "protein" gm. | P.c. N in "protein" prep. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vicia sp. (Vetch) | 30·0 | 11·0 | 26·7 | 5·10 | 1,362 | 1,124 | 1,119 | 23·9 | 4·68 |
| 19 | *Vicia dasycarpa* Ten. | 30·0 | 11·8 | 26·5 | 5·36 | 1,422 | 1,141 | 1,133 | 25·6 | 4·43 |

In Table 1 are shown the amounts of total N, coagulable N and protein N in seeds, the percentages of N in untreated seeds and in their "whole" protein preparations, and other data concerning the preparations.

*Chemical Methods.*

*Sulphur.* For estimation of cyst(e)ine (i.e. cystine plus cysteine, calculated as cystine) and methionine in "whole" protein preparations and the distribution of sulphur in the seeds Lugg's (1938) method was used, with the modified procedure of Lugg and Weller (1944) for total-S.

Throughout this work, the reagent sodium hydroxide was replaced with potassium hydroxide. With the use of KOH, although the ionic strength of the solution is the same, the salt effect on solubility of barium sulphate is greater than with the NaOH, as can be judged by the "blanks." The increase in solubility of $BaSO_4$ is detectable, but not sufficiently large to render negative the means of the random deviations of the blanks.

*Nitrogen.* For the nitrogen estimations a modified Kjeldahl method was adopted in which selenium-mercuric oxides catalyst was used (i.e. 40 mg. HgO with 40 mg. $SeO_2$ for the 0·3 gm. samples). The digest in the $H_2SO_4 - Na_2SO_4$ mixture was maintained at the boiling point for three hours after it had cleared. The made-up aliquots of the digest were distilled using Johanson's (1948a) modification of the micro-Kjeldahl apparatus, in which the distillation tube is surrounded by the steam jacket, which also serves as a "vacuum trap" for the spent liquor.

Cole and Parks (1946) have found that with a mercury-selenium catalyst, which was shown by Osborne and Krasnitz (1934) to be more efficient than the Se alone, the necessary time of digestion for the total conversion of N to ammonia even in ring compounds and high polymeric substances is reduced to 60 minutes, when a ratio of about 1:1 of the catalyst to nitrogenous compounds is used. Kaye and Weiner (1945) showed that with HgO alone

complete recovery of N was attained in resistant N ring compounds such as pyridine, pyrimidine, thiazole, pyrrole, quinoline and others, and White and Secor (1946) similarly showed for tryptophane and gramicidin that 60–90 minute digestion with a 5:1 ratio of HgO to compound was sufficient. Shirley and Becker (1945) found that Hg + $SeOCl_2$ catalyst completely converts N in nicotine, caffeine, pyridine, and quinoline into ammonia N in three hours after "clearing" (see also Clark, 1941). It is probable, therefore, that the combination of mercury-selenium will be more efficient as a catalyst for biological material such as seeds, than either HgO or $SeO_2$ alone. The use of mercury has been objected to because of the necessity of adding sulphide or thiosulphate before distillation, to decompose the Hg-ammonia complex, and of thoroughly washing out the apparatus each time to avoid accidental generation of hydrogen sulphide. The practice followed by Cole and Parks (1946), Clark (1941), and others, in adding thiosulphate or sulphide before commencement of distillation was found unnecessary in the presence of selenium and excess of alkali.

In Table 2, are given data showing complete recovery of the introduced N from the digest without the addition of sodium sulphide or thiosulphate.

The feasibility of replacing sulphur by selenium for the purpose of decomposing the supposed Hg-ammonia complex (see Neuberg, 1902) is not unexpected, in view of the similarity of Hg selenides to Hg sulphides.

The mean deviations in Table 2 clearly show that, in the presence of Se, ammonia is completely recovered from digests (b) and (c) containing mercury. Complete recovery of ammonia is attained in digest (a) with the addition of sodium thiosulphate, but it would appear, giving due regard to random errors, that even without this addition, the retention is small, being about 0·4 p.c. N in the aliquot when 10 ml. of 10 N KOH are used for 0·5 ml. of conc. sulphuric acid.

Since the period of digestion and amount of catalyst used by the various authors differ, it was preferred to check whether the deduced quantity of catalyst (i.e. HgO and $SeO_2$ 40 mg. of each) would suffice for the purpose.

A mixture of peas and straw, gave duplicates of 2·09(7) and 2·08(0) p.c. N, using the procedure of Johanson and Lugg (1946) in which $SeO_2$ was used as a catalyst and the digestion continued for eight hours after "clearing." This is the necessary time according to Chibnall (1943). The same sample, with HgO — $SeO_2$ as a catalyst, for duplicates gave 2·09(5) and 2·09(7) p.c. N while the digestion time after "clearing" was only three hours. Similarly, several other samples were subjected to even shorter periods of digestion, of two hours and less, with excellent agreement in duplicates. It seems, therefore, that the quantity of HgO — $SeO_2$ catalyst taken offers an opportunity to reduce digestion time, and ensures against losses through dissociation of ammonia from loss of $H_2SO_4$ on prolonged heating (Self, 1912).

## RESULTS.

In Table 3, the sulphur distributions in the "whole" protein preparations and seeds are shown as cyst(e)ine (cystine plus cysteine) and methionine, and expressed as percentages of the total nitrogen occurring in protein preparations and seeds samples. In conformity with the previous practice of Lugg and co-workers, the sulphate-S contents, too, have been expressed in relation to the N present.

The fundamental principles underlying these estimates by "differential oxidation" are extensively discussed by Lugg (1938, 1939b) and Lugg and Weller (1944). It need only be stated here that fundamental assumptions are made that the sulphur is present only as cyst(e)ine and methionine (free, or as amino acid residues in proteins) and sulphate, and that the amino acid residues behave with the reagents in the same way as the free amino

TABLE 2.

*Recovery of N in the presence of mercury and selenium.*
*Aliquot containing 0·504 mg. N, taken for each titre, representing 2·520 ml. HCl.*

| | (a) HgO 80 mg. | | | | (b) HgO 80 mg. + $SeO_2$ 20 mg. | | | | (c) HgO 40 mg. + $SeO_2$ 40 mg. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dist. No. | HCl ml. S.* | Deviation (×100) | HCl ml. | Deviation (×100) | HCl ml. S* | Deviation (×100) | HCl ml. | Deviation (×100) | HCl ml. S* | Deviation (×100) | HCl ml. | Deviation (×100) |
| (1) | 2·53(7) | 1·7 | 2·52(3) | 0·3 | 2·53(4) | 1·4 | 2·53(8) | 1·8 | 2·53(4) | 1·4 | 2·53(2) | 1·2 |
| (2) | 2·53(2) | 1·2 | 2·52(3) | 0·3 | 2·53(6) | 1·6 | 2·53(4) | 1·4 | 2·53(2) | 1·2 | 2·53(6) | 1·6 |
| (3) | 2·54(7) | 2·7 | 2·52(7) | 0·7 | 2·54(0) | 2·0 | 2·53(6) | 1·6 | 2·54(0) | 2·0 | 2·53(9) | 1·9 |
| (4) | 2·52(3) | 0·3 | 2·51(8) | −0·2 | 2·53(2) | 1·2 | 2·52(8) | 0·8 | | | | |
| Mean deviation | | 1·5 | | 0·3 | | 1·6 | | 1·4 | | 1·5 | | 1·6 |

S* represents distillations with addition of 2 ml. saturated $Na_2S_2O_3$ solution.

(Each digestion mixture contained 0·40 gm. glucose, 0·05 gm. NaCl, 2·50 gm. $Na_2SO_4$, 10 ml. conc. $H_2SO_4$, and 0·4756/5 gm. $(NH_4)_2SO_4$ in standard solution and a catalyst as indicated. Digestions continued for 1 hour after "clearing".)

acids. There is no evidence that such assumptions will not strictly hold for the "whole" protein preparations. In unextracted seeds, however, there is a distinct possibility of the values having been vitiated by inclusion of other compounds of S. To indicate this the values of cyst(e)ine and methionine content for the seeds are shown in italics. Notwithstanding its underlying specific assumptions, Lugg's (1938) "differential oxidation," on the basis of existing evidence, provides the most reliable method of estimation of small quantities of S-amino acids in samples (such as seeds) heavily contaminated with carbohydrate, polyuronide and cellulose impurities, where the colorimetric methods will fail to provide even a reasonable indication of the quantities of these acids present. (See Lugg, 1933, 1938, 1939b; Bailey, 1937). The inherent difference in the energy bonds of $H_3C$-S, H-S and S-S underlies the success of differential oxidation of S atoms in cyst(e)ine to sulphate, and not those of methionine. The values shown in Table 3 are the means of closely agreeing replicate estimations, whose random errors of deviations from the true means, for the upper limit of twice the "standard error," are believed not to exceed 0·01 for sulphate, 0·06 for methionine and 0·03 for cyst(e)ine in samples 1 and 19 and their "whole" protein preparations.

TABLE 3.

*Partial composition of the preparations: losses during extraction.*

| No. | Preparation from. | P.c. N. | P.c. Cyst. N. | P.c. Meth. N. | $\frac{SO_4 - S}{N} \times 100.$ | P.c. loss, or apparent loss, during extraction. Cyst. | Meth. | $SO_4$—S. |
|---|---|---|---|---|---|---|---|---|
| 1 | Vicia sp. (Vetch) seeds | *5·10* | *0·96* | *1·22* | 0·71 | 5 | 56 | 88 |
| 1 | Vicia sp. (Vetch) "whole" protein preparation | 4·68 | 1·11 | 0·69 | 0·10 | | | |
| 19 | *Vicia dasycarpa* Ten. seeds | *5·36* | *1·62* | *0·63* | 0·19 | 42 | 33 | —7 |
| 19 | *Vicia dasycarpa* Ten. "whole" protein preparation | 4·43 | 1·18 | 0·61 | 0·25 | | | |

On the right of Table 3 are shown the apparent losses of cyst(e)ine and methionine and the losses of sulphate during extractions in the preparation of "whole" protein samples. They have been calculated with the aid of the data shown in Table 1. Certain of the losses are qualified as "apparent," because as has been stated already, the cyst(e)ine and methionine values for seeds may have been vitiated by inclusion of other S-compounds.

In consideration of losses the random errors must be taken into account. Thus the random errors can account for the cyst(e)ine loss in Sample 1 but they are insufficient to account for the loss of cyst(e)ine in 19 and methionine in both 1 and 19. A different explanation must therefore be sought. It appears that Sample 1 has very little free cyst(e)ine, whereas either its free methionine content or some other S-compounds are high. Sample 19, on the other hand, is high in either free cyst(e)ine or free methionine or both, or in some other S-compounds.

## Discussion.

It is apparent from the data of Table 1 that the proportion of non-protein nitrogenous substances to the other extractable substances in the seeds of the vetch is large in comparison with some other legume seeds (see Johanson and Lugg, 1946; Lugg and Weller, 1944; Lugg and Clowes, 1945). The percentage of N in "whole" protein preparations is lower than in unextracted seeds. The data of Table 1 indicate also that the soluble and extractable nitrogenous substance or substances represent molecules of high N content in the case of Sample 19 and of somewhat lower N content in the case of 1.[4]

Further evidence of the unusual composition of vetch seeds is provided by Table 3. The S-distributions of the "whole" protein preparations are similar, but there is an extraordinary contrast in the S–distribution of the unextracted seeds. In this respect the vetch is entirely different from the soya bean (Johanson and Lugg, 1946), which showed very little variation, both in seeds and their "whole" protein preparations.

Before attempting to assess the value of vetch seeds as a source of S–containing amino acids, in comparison with other legume seeds, it is necessary to consider with some care the best method of expressing the results obtained. For instance, if the proportion of amino acid N to the total protein N is used, it is necessary to assume that the composition of proteins, and their relative proportions in seed material, are constant, or nearly so, under varying environmental conditions. If, on the other hand, the amino acid contents are expressed as percentages on the total dry weight of the seeds, it is necessary to make the additional assumption that the total protein content in relation to the other matter in seeds, such as starch, alkaloids, cellulose, etc., is somewhat "normal." ("Normal" is qualified here as the most frequently occurring ratio of total proteins to other substances within the environmental conditions only, and as the environmental conditions vary, the "normal" will also vary.)

With purified materials or impure protein preparations it is not only desirable but necessary to report amino acid contents on a N basis (see also Johanson and Lugg, 1946, footnote; Chibnall, 1945). The N values for the protein preparations should also be given, since arbitrary N contents for impure protein preparations are unsatisfactory (see Block and Bolling, 1945). Similarly, the amounts of protein preparations obtainable from a known quantity of starting material should also be given.

A protein preparation which is low in a particular amino acid may still give a higher total content of this acid in the original material, if it is available in larger quantities. Conversely, protein preparations with high amino acid content, but low "availability," may give low values expressed on the original material.

The significance of some of these points is illustrated in Table 4, where the cyst(e)ine and methionine contents of the seeds of several legumes are assembled.

[4] It is hoped to examine this question further.

TABLE 4.

*Comparison of cyst(e)ine and methionine contents of "whole" protein of leguminous seeds.*

| No. | Source. | Dry wt. seeds taken gm. | Dry wt. protein gm. | P.c. N. | P.c. Cyst. N of total N. | P.c. Meth. N of total N. | P.c. Cyst. N in seeds × 100. | P.c. Meth. N in seeds × 100. | Ratios to contents of peas. Cyst. | Meth. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Vicia sativa* (Vetch) | 26·7 | 23·9 | 4·68 | 1·11 | 0·69 | 4·65 | 2·89 | 1·76 | 1·24 |
| 19 | *Vicia dasycarpa* Ten. | 26·5 | 25·6 | 4·43 | 1·18 | 0·61 | 5·05 | 2·61 | 1·91 | 1·12 |
| a | Soya bean var. *Dunfield* | 35·3 | 19·4 | 9·39 | 1·01 | 0·88 | 5·21 | 4·54 | 1·97 | 1·96 |
| a | Soya bean var. *Lincoln* | 36·0 | 18·9 | 9·88 | 1·06 | 0·85 | 5·50 | 4·41 | 2·08 | 1·90 |
| b | *Pisum sativum* | 9·48 | 6·83 | 4·88 | 0·75 | 0·66 | 2·64 | 2·32 | 1·00 | 1·00 |
| b | *Lupinus angustifolius* ("embryos") | 6·15 | 4·30 | 9·01 | 1·42 | 0·26 | 8·95 | 1·64 | 3·39 | 0·71 |
| b | *Lupinus pilosus* ("embryos") | 11·5 | 7·76 | 9·57 | 1·56 | 0·48 | 10·1 | 3·10 | 3·82 | 1·34 |
| b | *Lupinus luteus* ("embryos") | 12·8 | 8·20 | 12·7 | 2·07 | 0·37 | 16·8 | 3·01 | 6·36 | 1·30 |
| c | *Trifolium subterraneum* (seeds) | 6·93 | 4·23 | 7·69 | 0·45 | 0·33 | 2·22* | 0·89* | 0·84* | 0·38* |
| c | *Trifolium subterraneum* (heads minus seeds) | 9·71 | 8·64 | 1·69 | 1·52 | 0·28 | | | | |

a, b, c values reported by Johanson and Lugg (1946): Lugg and Weller (1944): and Lugg and Clowes (1945) respectively.
* Calculated for 100 gm. of burr, i.e. seeds plus (heads minus seeds).

In columns 7 and 8 are given the percentages of the S–amino acid N contents of "whole" proteins of corresponding seeds; these were calculated using formula

$$\frac{(\text{p.c. N} \times \text{weight protein preparation} \times \text{p.c. amino acid N})}{\text{weight seeds taken}} \times 10^{-2}.$$

In columns 9 and 10 are given the ratios of cyst(e)ine and methionine of "whole" protein contents of corresponding seeds to those of peas.

In this Table the ratios of cyst(e)ine and methionine clearly show the similarity in protein S of the two representative species of the genus Vicia, as noted earlier. These vetches compare favourably with peas, and are an equal or better source of methionine, while their cyst(e)ine values are nearly twice that of peas. Their methionine contents are low in comparison with those of the soya bean, but the cyst(e)ine values are equally high. Even considering the sparing action of cyst(e)ine on methionine, it would appear that vetch seeds are not sufficiently high in S-amino acids to be an adequate diet for the rat, chick or man (see e.g. Glynn, Himsworth and Neuberger, 1945; Grau and Almquist, 1943; Rose, Haines and Johnson, 1942). However, non-toxic Vicia varieties may prove a valuable improvement on peas as a dietary supplement for stock, and may be of particular value for sheep in Western Australia.

The data of Table 4 also reveal the richness in protein S of lupin "embryos" (seeds minus testas). Thus the *L. angustifolius*, which is customarily regarded as being very low in methionine, improved its relative position, and its total protein S is twice that of peas. The "embryos" of *L. luteus* are outstanding in the cyst(e)ine contents of their proteins. The methionine content of both *L. luteus* and *L. pilosus* is higher than that of peas. It must be remembered, however, that whenever the nutritional values for the whole lupin seeds are considered, the given value in Table 4 for "embryos" should be multiplied by factors (derived from Lugg and Weller, 1944) 0·79, 0·71 and 0·74 for *L. angustifolius*, *L. pilosus* and *L. luteus* respectively. Incidentally, the contribution by testas to the total contents of the S-amino acids of lupins is very small (see Lugg and Weller, 1944) and is neglected in making this correction.

Stewart and Moir (1945) found that Merino wethers grew more wool on a lupin seed diet than on diets of pea seeds or Subterraneum clover seeds containing similar amounts of digestible protein. To determine whether this extra wool growth is associated with a large protein S content or the abundant presence of some other amino acid or acids in lupins, a further quantitative survey of amino acids in seeds of these legumes is necessary.

### Cyanogenesis and Toxicity of Genus Vicia (Vetches).

It is well established that the genus Vicia is not devoid of cyanogenetic glucosides, nor other toxic principles such as alkaloids. Bertrand (1906) describes vicianin, a cyanogenetic glucoside from *Vicia angustifolia*, and Wilczek and Tschumi (1919) claim it to be in *Vicia ervilia* as an active principle, Wernery (1929) mentions that seeds of different species of Vicia contain vicianin, from which HCN is liberated by enzyme action. Anderson, Howard and Simonsen (1925) find it (as well as the alkaloid vicin) in *Vicia sativa* L, and some other

reports on HCN in Vetch are given by Gortner (1920), Rosenthaler (1928), Guerin (1930) and others. Guerin (1930) states that it only occurs in seeds and not in other parts of the plant. There is also in Vetches the much studied alkaloid or N glucoside vicin, which on hydrolysis yields glucose and a base divicine (see Ritthausen, 1899; Levine and Senior, 1916; Fischer, 1914, and Winterstein, 1919), and it is possible that other alkaloids may be present (see Acton and Chopra, 1927).

From reports on HCN contents and feeding trials (Gortner, 1920; Bruyning and Haarst, 1900; Guerin, 1930 and others), there appears to be much uncertainty as to the degree of toxicity of Vetches (see also Steyn, 1933). The apparent contradictions are of interest since the HCN content may vary considerably in some plants, not only in the same species of plant growing in different localities, but also in the same plant at different times. This may also be true for other toxic principles (see also Steyn and Rimington, 1935). It also appears that some varieties or strains of Vetches are difficult to distinguish (Bailey, 1948) and the wrong identification may cause further uncertainty. Consequently, taxonomical classification is not sufficient, and chemical investigation on toxicity is necessary for locally grown strains.

In the present study, the intention was mainly to make a qualitative survey of HCN in the seeds of available Vicia (species and strains) and to separate them into groups on a chemical basis. These are listed in two groups in Table 5. Group 1 is the autocyanogenetic type, containing both the cyano-glucoside and the necessary enzyme to liberate the HCN, while under the same chemical test, Group II carries no HCN, or only a slight trace, and is classified as non-cyanogenetic.

*Method for Survey of HCN in Vetches.*

For qualitative determination of HCN, 1 gm. of dry seeds was quickly ground into flour in a mortar, and immediately transferred into a sterilized, stoppered 150 ml. erlenmeyer flask. Before commencement of the test, the seed flour was just damped with distilled water, then moistened picric acid paper was suspended just above the material from a looped glass rod and the flask stoppered. The picric acid papers were prepared as described by Skinner (1935). The seeds of *Vicia ervilia* 17 (Table 5), were subjected to an additional test. To 2 gm. of ground, moistened seeds, after 2 hours' maceration $NH_2SO_4$ was added and 60 ml. of distillate collected. This was tested for HCN with $AgNO_3$ and picric acid solution. No HCN could be detected.

## Discussion of HCN Tests.

The varieties in Group 1 showed a strong positive test and developed a brick red colour in 10 to 60 minutes from the time of moistening of ground seeds. Samples from Group 2 failed to change colour from pale yellow to pale orange in less than 8 hours and then remained pale orange for a further 24 or more hours (Samples 19, 20, 21, 22 and 23). In some cases the test continued from 3 to 4 days without the slightest change in the original colour of the test papers. It must be expected that with prolonged maceration, if only a small quantity of HCN is available, it will not be detected if there are also present in the seeds some substance or substances capable of combining with it (see also Rimington, 1932; Alsberg and Black, 1916). In practice, such HCN will have no significance.

Experiments were also carried out to test for the presence of cyano-glucosides which would not hydrolyse spontaneously when separated from the relevant enzymes. The results were negative. This is in agreement with existing literature, which provides no evidence of the separate occurrence of glucosides and hydrolysing enzymes in vetch seeds.

TABLE 5.

*Cyanogenesis in ripe seeds of genus Vicia grown in Western Australia.*

| | | Grown at. | Harvested. |
|---|---|---|---|
| | Group I. | | |
| 1 | *Vicia sativa* W150 | Muresk | December, 1946 |
| 2 | *Vicia sativa* W150 | Clackline | November, 1947 |
| 3 | *V. sp.* probably *sativa* | Perth | December, 1947 |
| 4 | *V. angustifolia* C.P.I. 10,005 | Perth | November, 1947 |
| 5 | *V. angustifolia* C.P.I. 10,005 | Perth | November, 1947 |
| 6 | *V. sativa* C.P.I. 10,005 | Perth | December, 1947 |
| 7 | *V. sativa* C.P.I. 9,996 | Perth | December, 1947 |
| 8 | *V. sativa* C.P.I. 10,007 | Perth | November, 1947 |
| 9 | *V. sp.* probably *angustifolia* | Perth | November, 1947 |
| 10 | *V. sp.* probably *sativa* | Perth | December, 1947 |
| | Group II. | | |
| 11 | *V. monantha* C.P.I. 9,187 | Perth | December, 1947 |
| 12 | *V. monantha* C.P.I. 9,187 | Clackline | November, 1947 |
| 13 | *V. monantha* C.P.I. 9,186 | Perth | December, 1947 |
| 14 | *V. disperma* C.P.I. 10,213 | Perth | December, 1947 |
| 15 | *V. graminea* C.P.I. 10,214 | Perth | November, 1947 |
| 16 | *V. peregrina* C.P.I. 10,006 | Perth | November, 1947 |
| 17 | *V. ervilia* C.P.I. 9,995 | Perth | December, 1947 |
| 18 | *V. sp.* similar to *dasycarpa* | Perth | December, 1947 |
| 19 | *V. dasycarpa* C.P.I. 9,189 | Muresk | December, 1946 |
| 20 | *V. dasycarpa* C.P.I. 9,189 | Perth | December, 1947 |
| 21 | *V. dasycarpa* C.P.I. 9,189 | Clackline | November, 1947 |
| 22 | *V. sp.* probably *angustifolia* | Guildford | November, 1947 |
| 23 | *V. sativa* C.P.I. 9,188 | Perth | December, 1947 |

The results from the samples in Group 2 (non-cyanogenetic) confirm the view that chemical investigations of toxicity are necessary. Sample 23, *Vicia sativa*, and Sample 22, *Vicia* (probably *angustifolia*), would on taxonomical classification be included in the Group 1, with the strongly autocyanogenetic varieties of *V. sativa* and *V. angustifolia*, whereas they showed only a trace of HCN similar to that of the *V. dasycarpa* type. *V. dasycarpa* 18 showed no HCN, and *V. dasycarpa* 19, 20 and 21 only a trace. *V. ervilia* 17, which is claimed to contain vicianin (Wilczek and Tschumi, 1919), provides a further example of either incorrect classification or extreme variability of this strain due to environmental influences. The Samples 1, 2, 19, 20 and 21 have an additional interest, as they represent varieties grown in different localities or years, but still persist in their autocyanogenetic and non-cyanogenetic grouping, although the actual quantity of HCN in the seeds may alter.

These results show that in the genus *Vicia*, chemical investigations, possibly supplemented, in special cases by feeding trials of varieties selected for suitability to local conditions, are essential.

SUMMARY.

The seeds of two Vicia species (the autocyanogenetic, *Vicia* sp. *sativa* and non-cyanogenetic, *Vicia dasycarpa* Ten.) have been examined with special regard to the sulphur distributions, and to the cyst(e)ine and methionine contents of the "whole" proteins.

The cyst(e)ine and methionine contents of the "whole" proteins in both Vicia species were found to be similar, while their sulphur distributions in the seeds were entirely different. The cyst(e)ine contents of vetch "whole" proteins are similar to those of soya bean, but the methionine contents are much smaller, being somewhat similar to those of peas (*Pisum sativum*).

Attention is drawn to the necessity of taking into account the "availability" of "whole" protein preparations in the original material before the comparison of amino acid values in various seeds could be made. The necessity for such comparison, which is based on actual weight of protein amino acids in original materials, is illustrated with the aid of the data in the form of a Table. The contents of sulphur amino acids in certain leguminous seeds have been revalued and new conclusions drawn.

The use of mercury-selenium catalyst was adopted for Kjeldahl nitrogen estimations, the maximum time of digestion being 2–3 hours. The addition of sulphides or thiosulphates to digest solutions to facilitate complete recovery of ammonia in the presence of mercury was found unnecessary if selenium is also present.

The survey of cyanogenesis of available seeds of Vicia was made, and classified into two groups, one, the autocyanogenetic, which contains cyanoglucoside and the necessary enzyme to liberate HCN, and the other non-cyanogenetic. The view is held that the chemical investigation of toxicity of genus *Vicia* is necessary, even with varieties definitely identified taxonomically and particularly after varieties are established and have adapted themselves to local conditions, before they can be classified as toxic or non-toxic.

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REFERENCES.

Acton, H. W. and Chopra, R. N. (1927): Bull. Hyg., 5, p. 620.

Alsberg, C. and Black, O. (1916): J. Biochem., 25, p. 133.

Anderson, L. A. P., Howard, A. and Simonsen, J. L. (1925): Ind. J. med. Res., 12, p. 613.

Bailey, E. T. (1948): Plant Introduction Officer, C.S.I.R.. Perth, W.A. (private communication).

Bailey, K. (1937): Biochem. J., 31, p. 1,396.

Bertrand, G. (1906): C. R. Soc. Biol., 143, p. 832.

Block, R. J. and Bolling, D. (1945): "The Amino Acid Composition of Proteins and Foods." Charles C. Thomas, Springfield, Ill., U.S.A.
Bruyning, F. F., Jun. and Haarst, J. van (1900): J. chem. Soc. abst., 1900, 2, p. 160.
Chibnall, A. C. (1943): Biochem. J., 37, p. 372.
Chibnall, A. C. (1945): 2nd Proctor Mem. Lecture, J. Internat. Soc. Leather Trades Chemists (1946), 30, p. 1.
Clark, E. P. (1941): J. Assoc. Official Agr. Chem., 24. p. 641.
Cole, J. O. and Parks, C. R. (1946): Ind. eng. Chem. Anal. Edn., 18, 1, p. 61.
Fischer, E. (1914): Ber., 47, p. 2,611.
Glynn, L. E., Himsworth, H. P. and Neuberger, A. (1945): Brit. exp. Path., 26, p. 326.
Gortner, A. R. (1920): Min. agr. exp. Sta. Breeders' Gazette, 77, p. 1,230 (C.A., 14, 2, 1862).
Grau, C. R. and Almquist, H. J. (1943): J. Nutrition, 26, p. 631.
Guerin, P. (1930): C. R. Soc. Biol., 190, p. 512.
Horn, M. J., Jones, D. B. and Ringel, S. J. (1942): J. biol. Chem., 144, p. 87.
Johanson, R. (1948a): Austral. chem. Inst. J. and Proc. (in press).
Johanson, R. (1948b): *ibid.* (in press).
Johanson, R. and Lugg, J. W. H. (1946): Austral. J. exp. Biol., 24, p. 321.
Kaye, I. A. and Weiner, N. (1945): Ind. eng. Chem. Anal. Edn., 17. p. 397.
Levine, P. A. and Senior, J. K. (1916): J. biol. Chem. 25, p. 607.
Lugg, J. W. H. (1933): Biochem. J., 27, p. 1,022.
Lugg, J. W. H. (1938): *Ibid.*, 32, p. 2,114.
Lugg, J. W. H. (1939a): *Ibid.*, 33, p. 110.
Lugg, J. W. H. (1939b): Appendix 1, "Protein Metabolism in the Plant" (A. C. Chibnall), Yale Univ. Press, U.S.A.
Lugg, J. W. H. and Clowes, G. J. (1945): Austral. J. exp. Biol., 23, p. 75.
Lugg, J. W. H. and Weller, R. A. (1944): *Ibid.*, 22, p. 149.
May, C. E. and Rose, E. R. (1922): J. biol. Chem., 54, p. 213.
Neuberg, C. (1902): Beitr. Chem. Physiol. Path., 2, p. 214 (J.C.S. Abst., 1902, 2, p. 426).
Osborne, R. A. and Kransnitz, A. (1934): J. Assoc. Official Agr. Chem., 17, p. 339.
Osborne, T. B. and Heyl, F. W. (1908): Amer. J. Physiol., 22, p. 423; (J.C.S. Abst. [1908]: 1, p. 843).
Rimington, C. (1932): Onderstepoort J.V.Sc. Animal Ind. 18th Edn., p. 955.
Ritthausen, H. (1899): J. chem. Soc. Abst., 1899, 1, p. 715.
Rose, W. C., Haines, W. J. and Johnson, J. E. (1942): J. biol. Chem., 1946, p. 683.
Rosenthaler, L. (1928): Pharm. Acta. Helv., 3, p. 31.
Schulze, E. (1893): Z. physiol. Chem., 17, p. 193; (J.C.S. abst. [1893]: 2, p. 85).
Self, P. A. W. (1912): Pharm. J., 34, p. 384.
Shirley, R. L. and Becker, W. W. (1945): Ind. eng. Chem. Anal. Edn., 17, p. 437.
Skinner, W. W. (1935): "Methods of Analysis of the Association of Official Agric. Chemists," Association of Official Agr. Chemists., Washington, D.C., U.S.A.
Stewart, A. M. and Moir, R. J. (1945): Report to Australian Wool Board, Jan., 1945.
Steyn, D. G. (1933): Onderstepoort, J. vet. Sci. Animal and Indust., I, p. 251.
Steyn, D. G. and Rimington, C. (1935): Onderstepoort J. vet. Sci. Animal and Indust., 4, p. 51.
Wernery, H. (1929): Inaug. Dissert., Dr. Med. Vet. Tierärztl., Hochschule, Hannover, 1929. (Cited by Steyn, D. G., "The Toxicology of Plants in South Africa," South Africa Central News Agency, Limited, 1934, South Africa.)
White, L. M. and Secor, G. E. (1946): Ind. eng. Chem. Anal. Edn., 18. p. 457.
Wilczek, E. and Tschumi, L. (1919): Lausanne. Schweiz. Apoth. Ztg., 57, p. 433, 488. (C.A., 13, 3, p. 2,971.)
Winterstein, E. (1919): Z. physiol. Chem., 105, p. 258.

# REGENERATION OF VIRUS RECEPTORS IN MOUSE LUNGS AFTER ARTIFICIAL DESTRUCTION

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Recent publications of Burnet and his school gave a full account of the properties of an enzyme found in the culture filtrates of *V. cholerae*. The effect of standard preparations (Stone, 1947a; Burnet and Stone, 1947) is identical on red cells (McCrea, 1947; Stone, 1947b), the respiratory lining of excised lungs (Fazekas de St.Groth, 1948) and glycoproteins of various origin which act as Francis inhibitors (Anderson and McCrea, 1948); the preparations render all these biological and chemical materials incapable of adsorbing viruses of the influenza group or release these viruses artificially from the adsorbed state. The "receptors" of virus adsorption are destroyed—at least in the biological sense—by their action. As the effectiveness of the preparations is not significantly reduced during prolonged and repeated action, the active principle is considered to be an enzyme, the "receptor destroying enzyme" (RDE) of *V. cholerae*.

The above-mentioned investigations made the therapeutic possibilities of RDE evident, as it is the "receptors" which would form the point at which the infective particle and the future host cell make their first contact. Unless there are receptor spots on the surface of the susceptible cell to which virus could be adsorbed, infection would not be realized. An extensive study was made by Stone (1948) on the prophylactic and therapeutic value of RDE. In the allantoic cavity a suitable dose of the enzyme—though it has no direct effect on the virus at all—prevents infection of the embryo to a significant degree, with some strains of virus against approximately 1,000 $LD_{50}$.

These findings undeniably bear out that RDE has essentially the same effect in the living organism as in lifeless models, and that in the absence of receptors the virus is incapable of establishing infection. However, the results obtained have by no means the all-or-nothing character of similar experiments carried out *in vitro*. The influence of standard doses of the enzyme varied widely according to the strain of virus used, the time elapsing between the administration of RDE and the dose of virus, and the order of administration. Relatively large amounts of enzyme were required, the effect diminishing disproportionately when the amount was reduced.

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The simplest explanation of these findings was to assume that while *in vitro* the number of receptors is constant, in the living organism there is a dynamic equilibrium maintained between decaying and newly-formed receptors. If large numbers are destroyed artificially, the organism compensates the loss by enhanced regeneration, the process being analogous to other types of physiological repair.

As the power of RDE and substances of similar action to reduce the number of receptors and keep it below the level necessary for infection is the *sine qua non* for any practical application in the prevention of influenza, it is of as much interest to know the response of the organism to receptor destruction, as the nature of receptor destruction itself. The present paper aims at elucidating this side of the question.

In developing chick embryos the regenerative processes can not be separated from the generative ones; the rate and extent of repair is not directly comparable with that of the adult mammalian organism; time is a serious limiting factor in work with eggs; and due to inherent technical difficulties it is almost impossible to obtain strictly quantitative results. All the experiments reported here were made on mice. These animals are susceptible to infection with the viruses of human influenza; time is no limiting factor in the experiments; and with methods devised and tested in previous work regeneration could be measured by simple and quantitative means.

## MATERIALS AND METHODS.

*Normal saline.* 8·50 gm. NaCl in 1,000 ml. distilled water.

*Citrate saline.* 20·0 gm. sodium citrate and 8·50 gm. NaCl in 1,000 ml. distilled water.

*Calcium saline.* 1·00 gm. $CaCl_2$, 8·50 gm. NaCl, 1·203 gm. $H_3BO_3$ and 0·052 gm. $Na_2B_4O_7.10\ H_2O$ in 1,000 ml. distilled water. The pH lay between 7·1 and 7·3.

*Red cells.* Blood was obtained from the wing veins of adult fowls, the cells washed in citrate saline and two changes of normal saline. Finally the cells were made up to 1 or 2 p.c. suspensions in normal saline. Between experiments the suspensions were kept in the refrigerator at 4° C., and no cells older than 36 hours were used.

*Virus.* The LEE strain of influenza B grown in fertile hen's eggs was used throughout the experiments. The harvested allantoic fluids were tested for their haemagglutinin titres and freedom from bacterial contamination. Those not contaminated and of titres higher than 500 against 1 p.c. fowl red cells were pooled and stored at 4° C.; they are referred to in this paper as "active". "Heated virus" was prepared by heating allantoic fluids containing active virus for 30 minutes at 56° C. in the water-bath. No allantoic fluid older than 12 days was used.

*Receptor destroying enzyme* (RDE). Plates of 1 p.c. agar were inoculated with a few drops of broth cultures of the "4Z" strain of *V. cholerae* and incubated for 16 hours at 37° C. The agar was removed into a gauze bag, and the fluid pressed through a Büchner funnel. The filtrate was left to settle in the refrigerator, and the supernatant removed. This fluid was heated in the presence of excess calcium at pH 6·0 at 55° C. for 40 minutes. The precipitate was removed by centrifugation. The fluid so obtained had a yellowish colour, was completely transparent, of a pH between 5·9 and 6·5. Its titre—as estimated by the standard technique—was about 1,500. These preparations were stored at 4° C., and are referred to in this paper as RDE.

For further details of the properties and preparation of RDE see Stone (1947b) and Burnet and Stone (1947).

*Anti-RDE rabbit serum.* This was from rabbits immunized with an emulsion of eluted enzyme, liquid paraffin and lanoline base. The properties of this serum are described in Burnet (1948).

*Titration of virus haemagglutinin.* The routine technique used in this laboratory for estimating haemagglutinin titres had to be slightly modified, as the samples containing virus contained in most cases RDE too. As the activity of RDE on red cells is greatly reduced in the absence of calcium ions, all dilutions were made in citrate saline. Virus suspensions gave the same titre in citrate saline as in normal saline, and 10 A.D. of virus could be accurately assayed in media containing 1,000 inhibitory doses of RDE.

Doubling dilutions were made in 0·25 ml. of citrate saline and 0·25 ml. of red cell suspension added. Readings were made an hour later by observing the pattern of the settled cells. Depending on the expected titre 1 or 2 p.c. red cell suspensions were used, but the titres are expressed always in terms of a 1 p.c. suspension. 1 A.D. is the amount of virus which causes "+" (partial) agglutination of 0·25 ml. 1 p.c. fowl cells. The accuracy of the method is approximately ± 25 p.c.

*Titration of RDE.* Doubling dilutions of the samples were made in 0·25 calcium saline and an equal amount of 1 p.c. cells added. The racks were thoroughly shaken and placed in a water-bath at 37° C. for 30 minutes. At the end of this time a drop (approximately 0·04 ml.) of active MEL A virus of a titre of 100 was added, the racks shaken and put back for another 30 minutes into the bath. The end-point was taken as the "+" pattern of settled cells.

*Treatment of mice.* 6–7 weeks-old laboratory mice of uniform breed were used in the experiments. RDE and anti-RDE serum were administered by *intranasal instillation* of 0·05 ml. fluid under light chloroform-ether anaesthesia, or inhaled in the form of fine droplets in the *mist apparatus.* The device was the same as used by Stone and Burnet (1945) in their experiments on the effect of halogens on virus mists, and full details are given in the cited paper. The apparatus is essentially a glass cylinder of 29 litres capacity with inlets for the mist and compressed air for clearing the chamber, and an outlet to a pressure equalizer. The RDE-containing fluid (titre 1,500) was vaporized in an atomizer under a pressure of 1·8 atm/cm$^2$, led into the mist chamber where an electric fan ensured thorough mixing throughout the time of the experiment. If not stated specially, the vaporizer was working 20 p.c. of the time of mist treatment, e.g. for three runs of one minute in an experiment of fifteen minutes or six runs of two minutes each in an hour.

*Preparation of mouse lungs.* The technique is fully described in a previous paper (Fazekas de St.Groth, 1948). In brief it consists of excising the lungs and slipping the trachea over a glass cannula inserted in a one-hole rubber stopper. The stopper is placed in a large test tube with a side arm through which the pressure inside the vessel can be regulated. If fluids are measured into the cannula and the pressure in the container lowered, they are aspirated by the lungs and by raising the pressure they can be pressed out again. By this method the respiratory surface can be washed at suitable intervals and the washing fluids tested. When virus containing allantoic fluid is introduced into the lungs the virus is adsorbed to the respiratory lining and subsequent washings at first contain no detectable agglutinin. After a certain time the virus elutes and measurable amounts of agglutinin appear in the washings. If the total amount of agglutinin regained in the washing fluids (free agglutinin) is plotted against time an adsorption-elution curve is obtained. This can be regarded as essentially the converse of a curve indicating the amount of virus (haemagglutinin) still retained on the lung surfaces. The comparison of such curves forms the subject of this study.

To stop the action of any RDE present in the lungs, previous to experiments all lungs were washed with three changes of citrate saline. Then 0·5 ml. of the virus-containing allantoic fluid diluted if necessary to 1,000 A.Ds was aspirated and left in contact with the respiratory tract for 10 minutes. At the end of this period it was pressed out, a sample of 0·05 ml. quickly taken and the fluid run back into the lung for another 10 minutes after which it was removed. The curves shown in the Figures represent the additive recovery of virus as free agglutinin from this point onwards. These subsequent samples were obtained by the introduction and subsequent withdrawal after one minute of 0·5 ml. of normal saline at 10-minute intervals in the first hour, at 15 or 20 minutes in the second hour, and twice in the third. To check the quantity of virus used, the lungs were treated with RDE for 15 minutes after the conclusion of the experiments and by this means all of the virus was liberated.

The variations in results obtained by this method fall apparently in a narrower range than the accuracy of a single agglutinin titration. But it has to be borne in mind that the final titre is the sum of at least twelve determinations. Each of these titrations has an error of ± 25 p.c. but the random distribution of errors tends to decrease the final error. Thus the final values have to be considered as *means of twelve determinations* and this in turn explains the unusual reproducibility of results by this biological method.

## PRELIMINARY EXPERIMENTS AND DISCUSSION OF THE METHOD.

In previous work it was found that the time for which a certain dose of virus stayed adsorbed to the lung varied with the amount of virus used in the test. Following up this lead the phenomenon of adsorption-elution was studied in some detail. It was found that the time between the administration of the virus and its spontaneous elution in the excised lung was inversely proportional to the quantity of virus used. On the evidence of other experiments it appears that the amount of substrate for the virus-enzyme or RDE of *V. cholerae* varies only between narrow limits in the lungs of normal mice, and the findings can be explained on the basis of the Michaelis-Menten equation as an enzyme action in the presence of excess substrate. The period of "adsorption" is the "zero order phase" of the reaction, when all of the enzyme is bound and the alteration of the substrate proceeds at maximum speed. In this phase the rate of decrease of available substrate depends only on the amount of active enzyme present. In the second phase, as the concentration of substrate is sufficiently reduced the rate of the reaction decreases progressively, and a fraction of the enzyme is not held in enzyme-substrate complex. At this stage in experiments with virus "spontaneous elution" is observed. In the last phase most of the substrate has already been used up, the probability of successful collisions is very low; the rate of the reaction approaches asymptotically the zero level and practically all of the enzyme is free.

If this explanation is adopted as a working hypothesis, it is evident that by the use of a standard dose of virus or of any receptor destroying enzyme the amount of substrate (receptors) can be approximately measured. It will be directly proportional to the time needed for the virus to elute. This was the

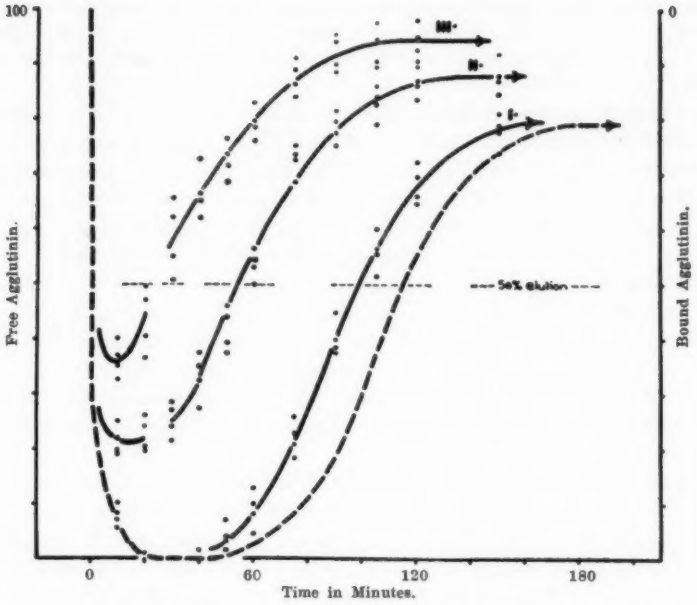


**Fig. 1.** *Adsorption-elution curves of a standard virus dose in excised mouse lungs after enzymatic destruction of receptors.*

- - - - Untreated control lungs.

I. Lungs treated previously with RDE 1:40 for 6 minutes, or RDE 1·80 for 12 minutes.

II. Lungs treated previously with RDE 1:40 for 12 minutes.

III. Lungs treated previously with RDE 1:40 for 24 minutes, or RDE 1:80 for 12 minutes.

Ordinates represent for the first portion of the curve the percentage of haemagglutinin still free in lungs to which 0·5 ml. fluid containing 1,100 haemagglutinating doses had been added.

The second portion from 20 minutes onward is constructed by successive addition of amounts of free agglutinin obtained at each washing, and represents essentially the amount of agglutinin still bound by the lung as indicated by the reversed scale on the right.

The method of assessing $T_{50}$ is shown in the Figure. It will be seen that this measure becomes unreliable at values of 25 minutes or less.

principle underlying the methods used throughout the experiments reported in this paper. To establish the possibility of determining the number of receptors by this means and to test the reliability and accuracy of the technique a few preliminary experiments were performed on excised lungs.

In the first series the excised lungs were treated with 0·5 ml. of dilute RDE giving a titre of 50 by the standard technique. The time of action was 6–12–24 minutes respectively in the three experimental groups. At the end of this period the enzyme was removed and the

respiratory tract washed with anti-RDE serum followed by three changes of citrate saline. Subsequently 1,100 A.D. of active LEE virus contained in 0·5 ml. of allantoic fluid were aspirated into the lungs and the adsorption-elution curves determined by the technique described under Methods.

As the most convenient measure of the time taken by the virus to elute, the point on the adsorption-elution curve at which 50 p.c. of the standard dose had been recovered "$T_{50}$" is adopted. As at this time the enzyme reaction is already proceeding at less than maximum speed this concept is only useful provided no theoretical conclusions are drawn from it. In this paper it is retained simply as a practical method of comparing processes by the comparison of a single characteristic.

The result of these preliminary experiments is shown in Fig. 1.

As compared with the untreated control lungs the standard dose of virus was adsorbed for a significantly shorter time to lungs pre-treated with RDE. The $T_{50}$ values of lungs treated for 24–12–6 minutes are 26–54–97 respectively, while the untreated normal lungs have the value of 112 with the amount of virus used in this test.

In a second experiment the corollary of the assumption was tested: whether appropriate dilutions of RDE acting for a standard time would produce identical effects with those related above. Dilutions with titres of 25, 50 and 100 were used and kept in the lungs for 12 minutes. Fig. 1 shows the results of this experiment, too. There is no doubt that by variation of either time or the amount of enzyme used, significantly different adsorption-elution curves are obtained with subsequently administered standard doses of virus. It is borne out with equal clarity that the product of enzyme quantity and time of action gives a constant, and the change in the number of available receptors can be measured and expressed satisfactorily in terms of adsorption-elution of a standard dose of virus.

When there is little or no excess of substrate, i.e. in cases where the preliminary adsorption of the test dose of virus is incomplete, $T_{50}$ cannot be directly measured. An approximate measure of receptor content is, however, given by the degree to which the level of free agglutinin is reduced at the 20 minute sample. Values below 32 minutes (25 p.c.) are approximations derived from a consideration of this level plus the subsequent form of the curve.

## Experimental Results.

*The Effect of RDE Inhalation.*

In the *first series of experiments* on living mice, the animals were kept in the mist apparatus for 15, 60 or 120 minutes and inhaled RDE in the form of fine droplets. At various intervals ranging from 2–120 hours after the treatment a number of mice was sacrificed, their lungs excised and prepared for the assay as quickly as possible. The difference in time between two parallels never exceeded three minutes. Immediately after

preparation the respiratory tracts were rinsed with three changes of citrate-saline to stop further action of the enzyme. The period between the half-time of mist treatment and the third washing with citrate was arbitrarily taken as the time of RDE action.

In lungs excised two hours after RDE inhalation there was a marked difference in the number of available receptors. The 50 p.c. elution times seem to be roughly in inverse proportion to the time of RDE administration. A protocol of a typical experiment is given in Table 1.

TABLE 1.

*Effect of previous RDE inhalation on virus adsorption—elution in excised lungs.*

| GROUP | DURATION OF RDE INHALATION | ADSORPTION EXPERIMENT | | | | | | | | | | | | | | $T_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Time of sampling ( minutes ) | | | | | | | | | | | | | |
| | | | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 75 | 90 | 105 | 120 | 150 | 180 | RDE | |
| I. 1 | 0 minutes | agglutinin titer of sample | 500 | 15 | 0 | 0 | 0 | 0 | 20 | 30 | 50 | 50 | 80 | 160 | 25 | 120 | 126 |
| 2 | | | 500 | 15 | 0 | 0 | 0 | 0 | 15 | 25 | 25 | 35 | 100 | 160 | 40 | 80 | 129 |
| 3 | | | 500 | 50 | 0 | 0 | 0 | 15 | 10 | 15 | 20 | 50 | 80 | 140 | 30 | 120 | 133 |
| 4 | | | 500 | 10 | 0 | 0 | 0 | 10 | 15 | 25 | 30 | 40 | 120 | 100 | 50 | 160 | 124 |
| 5 | | | 500 | 25 | 0 | 0 | 0 | 5 | 15 | 30 | 50 | 40 | 100 | 100 | 60 | 80 | 123 |
| 6 | | | 500 | 40 | 0 | 0 | 0 | 0 | 5 | 10 | 25 | 30 | 120 | 160 | 40 | 120 | 132 |
| | | average | 500 | 23 | 0 | 0 | 0 | 5 | 13 | 22 | 30 | 41 | 100 | 136 | 41 | 114 | 128 ± 4 |
| | | % free agglutinin | 100 | 6 | 0 | 0 | 0 | 1 | 4 | 8 | 14 | 22 | 42 | 69 | 78 | 100 | |
| II. 1 | 15 minutes | agglutinin titer of sample | 500 | 25 | 0 | 0 | 0 | 5 | 15 | 30 | 30 | 40 | 80 | 120 | 40 | 180 | 132 |
| 2 | | | 500 | 15 | 0 | 0 | 5 | 20 | 30 | 35 | 50 | 70 | 100 | 50 | 30 | 100 | 111 |
| 3 | | | 500 | 40 | 0 | 0 | 0 | 0 | 5 | 20 | 30 | 30 | 100 | 160 | 60 | 60 | 132 |
| 4 | | | 500 | 30 | 0 | 0 | 0 | 10 | 25 | 35 | 40 | 60 | 100 | 100 | 60 | 80 | 117 |
| | | average | 500 | 28 | 0 | 0 | 1 | 9 | 19 | 30 | 37 | 50 | 95 | 108 | 47 | 95 | 123 ± 6 |
| | | % free agglutinin | 100 | 6 | 0 | 0 | 0 | 2 | 6 | 12 | 15 | 29 | 48 | 70 | 79 | 98 | 11 |
| III. 1 | 60 minutes | agglutinin titer of sample | 500 | 60 | 70 | 15 | 15 | 25 | 35 | 80 | 60 | 30 | 30 | 10 | 10 | 100 | 76 |
| 2 | | | 500 | 100 | 80 | 10 | 10 | 20 | 40 | 80 | 80 | 40 | 30 | 20 | 15 | 100 | 78 |
| 3 | | | 500 | 100 | 100 | 5 | 20 | 30 | 50 | 60 | 70 | 30 | 30 | 15 | 10 | 70 | 68 |
| 4 | | | 500 | 100 | 80 | 10 | 20 | 30 | 60 | 70 | 70 | 25 | 20 | 15 | 10 | 80 | 71 |
| | | average | 500 | 95 | 83 | 10 | 16 | 26 | 46 | 78 | 75 | 36 | 28 | 15 | 11 | 88 | 73 ± 4 |
| | | % free agglutinin | 100 | 19 | 17 | 19 | 32 | 27 | 37 | 52 | 67 | 74 | 80 | 83 | 85 | 103 | 4 |
| IV. 1 | 120 minutes | agglutinin titer of sample | 500 | 140 | 150 | 80 | 40 | 60 | 35 | 40 | 20 | 20 | 20 | 10 | 9 | 80 | 45 |
| 2 | | | 500 | 160 | 180 | 35 | 50 | 50 | 30 | 30 | 20 | 15 | 10 | 10 | 10 | 60 | 37 |
| 3 | | | 500 | 120 | 140 | 25 | 30 | 50 | 40 | 40 | 25 | 20 | 20 | 15 | 10 | 80 | 49 |
| 4 | | | 500 | 160 | 170 | 30 | 70 | 40 | 30 | 30 | 20 | 20 | 10 | 5 | 0 | 80 | 37 |
| | | average | 500 | 145 | 160 | 28 | 52 | 48 | 34 | 35 | 21 | 19 | 15 | 10 | 5 | 80 | 42 ± 6 |
| | | % free agglutinin | 100 | 28 | 32 | 38 | 48 | 58 | 64 | 71 | 76 | 79 | 82 | 84 | 85 | 102 | |

(Interval between mist inhalation and the excision of lungs 120 minutes.)

It is to be seen that the group of mice which inhaled RDE only for 15 minutes differs very slightly and by no means significantly from the untreated controls after two hours. On the other hand there is a striking reduction in the $T_{50}$ values of the groups treated for 60 and 120 minutes respectively. The 50 p.c. elution times are in this experiment 0:15:60:120 = 128:123:73:42, or expressed in percentage of the untreated controls 100:96:57:33.

Animals killed six hours after RDE administration show further significant reduction in the number of available receptors, the drop being equally apparent even in the group treated only for 15 minutes. The action of RDE is progressive and continues to destroy receptors at a steady rate: there is a marked decrease of the $T_{50}$ values in all groups between the second and sixth hour after treatment with the enzyme preparation.

From the eighth hour onwards there is no gross change in the number of receptors till approximately the twenty-eighth hour. It is of interest to note that the minimal $T_{50}$ values are very nearly the same in the groups treated with RDE for 60 or 120 minutes, while mice which inhaled RDE for 15 minutes show a definitely higher $T_{50}$ value.

After the period of decrease in the $T_{50}$ time (0–8 hours) and the stagnation at a minimal level (8–28 hours), a steady rise in the number of receptors begins. The 50 p.c. elution time in the end is very nearly the same as the normal. The experimental data are summarized in Table 2. All experiments were done in quadruplicate and the $T_{50}$ values used were the arithmetical means ± standard deviations of the four estimations. Percentage is calculated on the basis of the normal $T_{50}$ in the standard experiments (128 ± 4 minutes), the formula being $\% \ T_{50} = \frac{100 \times T_{50}}{128}$.

In view of the difficulty of giving an accurate quantitative interpretation to curves obtained with incomplete primary adsorption of virus this and similar Tables have been deliberately simplified by expressing the percentages to the nearest 5 and by using only "0" where there was virtually no adsorption of virus and "25 p.c." for minor degrees of adsorption. This is sufficient to establish all the significant experimental findings. The detailed findings are maintained on file in the Institute.

TABLE 2.

*50 p.c. elution times ($T_{50}$) after the inhalation of RDE-mist.*

100 p.c. $T_{50}$ = 128 minutes.

| Group. | Duration of treatment. | Time in hours between mist inhalation and excision of lungs. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 6 | 8 | 16 | 24 | 30 | 38 | 48 | 60 | 72 | 96 | 120 |
| I. | 15 minutes | 95 | 65 | 55 | 60 | 55 | 60 | | 75 | 80 | 90 | 100 | 100 |
| II. | 60 minutes | 60 | 30 | 25 | <25 | 25 | 25 | 40 | 50 | | 80 | 95 | 95 |
| III. | 120 minutes | 35 | 25 | <25 | 25 | 25 | 30 | | 50 | 75 | 85 | 95 | 100 |

The percentages of $T_{50}$ are stated to the nearest 5.

Though the results leave very little doubt as to the final number of newly-formed receptors, some lungs were tested 8, 11 and 14 days after RDE treatment. As expected the regeneration curve tends to the normal $T_{50}$ value and reaches it between the fifth and eighth day. Though the deviations from the normal were greater than in untreated animals, ranging from 89 to 116 p.c. as compared with 97–105, the average $T_{50}$ after 14 days was again 100 ± 6 p.c.

In the *second series of RDE inhalation experiments* the animals were treated in the mist chamber for the standard time of 60 minutes on 14 consecutive days. Two mice were sacrificed each day two hours after the last treatment and the excised lungs tested for available receptors. The results of the first five days are graphically recorded in Fig. 2. It is to be seen that the repeated administration of RDE causes a progressive decrease in the number of available receptors. The minimum, zero value as estimated by the technique used, is reached on the fifth day. From then on there is no change throughout the 14 days of the experiment: neither active nor heated virus is adsorbed to the excised lungs.

During the first five days the number of receptors is roughly in inverse proportion with the number of RDE inhalations. This, of course, does not hold for the first day's result, but it has to be borne in mind that the effect of RDE in these experiments was estimated two hours after its administration, while the maximum effect is reached only in the eighth hour, as reported above.

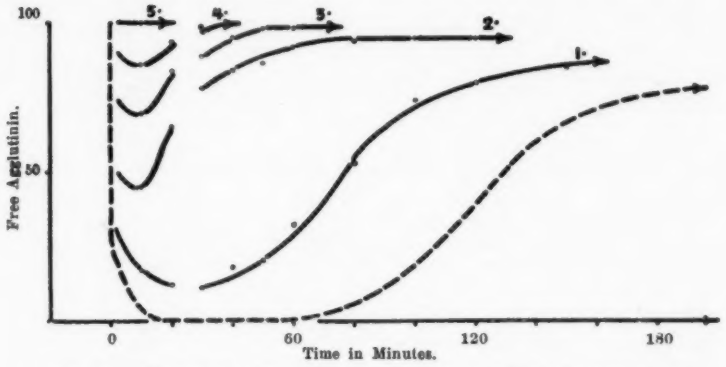


**Fig. 2.** *Adsorption-elution curves after prolonged RDE treatment.*
RDE-mist was inhaled by living mice for 60 minutes on five consecutive days. The number on the curves indicates the number of treatments.
- - - - Untreated control lungs.

The regeneration of receptors after prolonged treatment with RDE was studied on two groups of mice. The first inhaled RDE-mist on six successive days and the animals were killed at intervals ranging from 2–120 hours after the last treatment. The second group was treated for 14 days and the excised lungs tested at the same intervals. As the change in the $T_{50}$ values was not finished by the fifth day, when the experiment was repeated groups of mice were killed and the lungs tested 6–7–8–10–12–14 days after the last treatment. The results are given in Table 3. For the sake of comparison the second row of Table 2 is included: the $T_{50}$ values of a group of mice treated once for 60 minutes with RDE-mist.

TABLE 3.

*50 p.c. elution times ($T_{50}$) after prolonged RDE treatment.*

100 p.c. $T_{50}$ = 128 minutes.

| Group. | Length of treatment. | Time after last treatment. Hours. 2 | 8 | 24 | 30 | Days. 2 | 3 | 4 | 5 | 6 | 7 | 8 | 10 | 12 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I. | 1 × 60 minutes | 60 | 25 | 25 | 25 | 50 | 80 | 95 | 95 | 100 | 100 | 100 | 100 | 100 | 100 |
| II. | 6 × 60 minutes | 0 | 0 | 0 | 0 | <25 | 50 | 100 | 125 | 130 | 140 | 145 | 140 | 130 | 120 |
| III. | 14 × 60 minutes | 0 | 0 | 0 | 0 | <25 | 55 | 105 | 125 | 135 | 145 | 145 | 140 | 135 | 125 |

The percentages of $T_{50}$ are stated to the nearest 5.

Apparently there is no gross difference between the rate of regeneration after 6 or 14 days' consecutive treatment. Both start from a zero level, the $T_{50}$ reaches directly measurable values at approximately the thirty-sixth hour, and the normal value is reached between the fourth and fifth day. But when compared with the regeneration after a single dose of RDE,

these groups are markedly different. Though the starting value after prolonged treatment is significantly lower and measurable regeneration appears somewhat later, the normal level of receptors is reached sooner: *the slope of the regeneration curve is steeper.* The second difference is in the number of newly-formed receptors. After a single treatment the $T_{50}$ values converge asymptotically to a limiting maximum which coincides with or is slightly higher than that of untreated control mice. Even if the values were above the norm the difference was never found to be greater than 15 p.c., and 14 days after the last treatment, than 5 p.c. On the other hand *regeneration after prolonged RDE treatment* is definitely of the *hypercompensative* type. Maximal values found between the seventh and tenth day are in the average 42 p.c. above the norm, and even a fortnight after the last treatment the $T_{50}$ values are $25 \pm 7$ p.c. higher than those of mice treated at one occasion with RDE. The theoretical and practical significance of these findings is discussed below.

*Effects of Intranasal Instillation of RDE.*

As by the experiments reported above the effectiveness of RDE inhaled in the form of mist—a possible means of administration in human prophylaxis—was established, the rest of the work made use of the simpler and more quantitative technique of intranasal instillation of RDE solutions.

At first the experiments performed with RDE-mist were repeated. Using various dilutions of the enzyme preparation it was found that the effectiveness of intranasally-instilled RDE as compared with the effect of similar quantities on excised lungs is only 15–20 p.c. This finding can be explained by the presence of "receptors" in the upper respiratory tract, by the fact that nasal secretions contain a substrate for the receptor destroying enzyme (Burnet and Stone, 1948), and that there is always the possibility of the inoculum being partly swallowed. In turn the fact that at least 80 p.c. of the intranasal inoculum is lost may help to a better understanding of the general finding that viruses of the influenza group when titrated in mice give lower infectivity end-points than those obtained by titrations in developing eggs.

It was found further that 0·05 ml. of an RDE dilution giving a titre of 600 by the standard technique used in this laboratory had the same effect as the inhalation of RDE mist for 60 minutes. As the titre of the vaporized fluid was 1,500, and the amount of active RDE in the lung after intranasal instillation is not more than 1/5 of the inoculum, it is computed that in 60 minutes 0·004 ml. of the mist got down into the lungs of the mice.

A detailed relation of the experimental findings does not seem to be necessary as the results were essentially identical with those reported in the previous section. It is of interest though that if a heavy inoculum of RDE (concentrated by adsorption-elution on red cells after the method of Burnet and Stone (1947), titre 8,000) was used, it reduced the number of receptors to zero for 24 hours, and the subsequent regeneration was of the hypercompensative type. The rate of regeneration was enhanced and the final number of receptors formed was significantly above the normal ($123 \pm 11$ p.c.), though less than after prolonged daily treatment with RDE-mist. However, even in this case there was no appreciable difference in the $T_{50}$ value 14 days after the instillation of RDE.

In these experiments again the number of available receptors dropped steadily for the first six hours, slowed down and stayed at a minimal level from the eighth to approximately the twenty-fourth hour when the rise began. To decide whether (1) the effectiveness of RDE is lost after 8 hours and regeneration starts only after 24, or (2) RDE is persisting in the lung and destroying newly-formed receptors, its action being overcome by regeneration around the twenty-fourth hour, the following experiments were performed. The standard amount of 0·05 ml. of RDE (titre 1,500) was used as inoculum. A certain time after its administra-

tion 0·05 ml. of a potent anti-RDE rabbit serum was instilled intranasally. By this means the time of RDE action could be strictly limited, and the two overlapping phenomena of destruction and regeneration of receptors neatly separated. The final results of this experiment are to be seen in Table 4.

TABLE 4.

*50 p.c. elution times ($T_{50}$) following RDE treatments of controlled duration.*

100 p.c. $T_{50}$ = 128 minutes.

| Group. | Length of RDE action. | Time in hours between RDE treatment and excision of lungs. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 8 | 16 | 24 | 30 | 48 | 72 | 96 |
| I. | 1 hour | 60 | 65 | 70 | 80 | 90 | 95 | 95 |
| II. | 2 hours | 30 | 35 | 45 | 60 | 75 | 90 | 95 |
| III. | 3 hours | <25 | <25 | 40 | 50 | 65 | 80 | 95 |
| IV. | 6 hours | 0 | <25 | 25 | 40 | 60 | 85 | 90 |
| V. | 8 hours | 0 | <25 | <25 | 30 | 60 | 75 | 90 |
| VI. | 16 hours | | 0 | <25 | 25 | 55 | 80 | 95 |
| VII. | 24 hours | | | 0 | <25 | 50 | 80 | 90 |
| VIII. | 48 hours | | | | | 50 | 75 | 90 |
| IX. | 72 hours | | | | | | 75 | 95 |
| X. | anti-RDE untreated control | 0 | 0 | 0 | <25 | 50 | 75 | 90 |

The percentages of $T_{50}$ are stated to the nearest 5.

Length of RDE action = time between the administration of RDE and anti-RDE.

If the action of RDE is restricted to a time less than eight hours, the degree of receptor destruction is directly proportional with the time of action. In the absence of active RDE, regeneration of destroyed receptors is measurable from the eighth hour onwards. If the antiserum is given between the eighth and twenty-fourth hour, very shortly afterwards newly-formed receptors appear. From the thirty-sixth hour on the administration of anti-RDE serum does not influence the number of receptors at all.

These results bear out that RDE by virtue of its continued enzymatic action is capable for a certain time of maintaining a dynamic equilibrium with regeneration by destruction of newly-formed receptors. Computing the amount of active enzyme in the respiratory tract after eight hours from the delay caused in the rise of $T_{50}$, it is found to be less than 5 p.c. of the originally administered quantity. After twenty-four hours there is no appreciable amount of active enzyme left in the lung. This suggests that RDE is either inactivated in or eliminated from the respiratory tract. The latter possibility is proven to be the case by the experiments: (1) neither the washing fluids nor the extracts of ground lungs contained substances which would inactivate the enzyme; (2) small daily doses of RDE are as effective on the fourteenth day of successive treatment as on the first; (3) as shown by Burnet and Stone (1948) it is not only the receptors of the respiratory lining which contain the substrate for the enzyme but the secreted and eliminated mucus too, by which RDE could be mopped up and mechanically removed.

By neutralizing RDE after a short time the appearance of newly-formed receptors is not masked by the counteraction of the enzyme. Fig. 3 gives the adsorption-elution curves of lungs treated for three hours with RDE and tested for available receptors 6–96 hours after treatment. This might be considered as the standard picture of the response after artificial destruction of virus receptors in living mice.

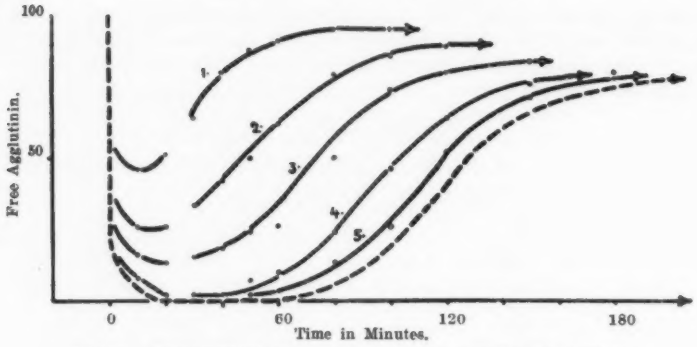


**Fig. 3.** *Regeneration of receptors after enzymatic destruction.*

Mice were treated with 0·05 ml. RDE intranasally followed by anti-RDE serum three hours later.

The adsorption-elution curves were determined: (1) 6 hours, (2) 24 hours, (3) 48 hours, (4) 72 hours, (5) 96 hours after the administration of RDE.

- - - - Untreated control lungs.

None of the mice used died during the time of the experiments, and there were no pathological signs apparent.

The histological examination of RDE-treated lungs did not reveal striking changes. After intranasal instillation of the enzyme preparation or after inhalation of RDE mist there was a marked active hyperaemia of the lungs, occasionally with microscopic interstitial haemorrhages and subpleural petechiae. The preparations from control mice treated with normal saline instead of RDE showed similar congestion, the difference in degree being not significant, though suffusions were not observed.

In many preparations there was a suggestion of the metachromatic property of respiratory mucus having been lost after the action of the enzyme. Nevertheless, the findings are not sufficient to draw any conclusions from them, but they call for further study of the effect of RDE on the histochemical properties of mucins.

## DISCUSSION.

The work reported in this paper is in accord with all the previous work from this laboratory in indicating that both RDE and virus act in typically enzyme fashion in the phenomena under study. Their effectiveness is not lost during prolonged or repeated action and the kinetics of the reactions are in good accordance with the Michaelis-Menten equation. On this assumption the time between the adsorption of a standard dose of active virus to the respiratory lining and its spontaneous elution is directly proportional to the number of available receptors. A detailed analysis of the kinetics of receptor destruction will be given in a separate paper. In the evaluation of experimental findings reported in the present paper attention was almost wholly confined to the directly measurable 50 p.c. elution time ($T_{50}$), and this was taken as an index of the number of receptors.

In normal mouse lungs with the virus dose used throughout in the experiments, $T_{50}$ is 128 minutes. By the action of RDE in living mice there is a sharp and significant drop in $T_{50}$. During the first six hours the values are roughly in inverse proportion to the time elapsing between the administration of RDE and the experimental estimation of receptors in the excised lung. If the action of the enzyme is limited to a certain time (e.g. 3 hours in the reported experiments) and stopped by the end of this period by the intranasal instillation of a potent anti-RDE serum, there is no appreciable change in the $T_{50}$ values to the eighth hour. From then on the 50 p.c. elution time rises progressively and approaches asymptotically a maximum coinciding with or slightly above the normal. Practically complete regeneration of receptors after a dose of RDE which reduces the normal $T_{50}$ to one-twentieth of its original value takes approximately six days. After 14 days there is no detectable difference between the 50 p.c. elution times of untreated normal mice and those whose receptor level has been reduced by RDE to 5 p.c. of the normal.

Administration of enzyme intranasally or by mist inhalation in the living animal is by no means exactly equivalent to the introduction of enzyme into the excised lung. In the first place a much smaller volume of fluid is used and this will be much less uniformly distributed over the respiratory surface; some will be swallowed and—due to the small volume—a relatively large quantity of active substance lost in the nasal passages. A comparison of the maximal effect produced by a given dose in the intact animal with graded amounts in the excised lung preparation indicates that RDE *in vivo* has only about 15 p.c. the activity of the same dose *in vitro*. The progressive removal of receptors in the first six hours after administration shows that the process is an enzymic one with continuous reliberation of RDE which is then available for further action. This is confirmed by the fact that anti-RDE given a certain time after RDE prevents subsequent destruction of receptors.

Like any other foreign material, however, RDE is progressively eliminated from the lungs. The route and means of this elimination have not been fully determined, but it is apparently completed within 24 hours since intranasal instillation of anti-RDE 24, 48 or 72 hours afterwards has no detectable influence on the regeneration of receptors. Burnet and Stone (1948) have shown that human respiratory mucus contains a substrate for RDE, and in previous work it was seen that there are measurable amounts of similar substrate in mouse lung washings. It is reasonable therefore to assume that much of the RDE may be eliminated while united with secreted mucus which is continuously removed by ciliary action.

The loss of enzyme activity after a certain time can not be due to some specific immunological neutralization, as it has been shown that doses of RDE are equally effective on the first and fourteenth day of continuous treatment. Daily doses of RDE have actually a cumulative effect by which the $T_{50}$ (i.e. the

number of receptors) can be reduced to and kept at a zero level even for as long a period as fifteen days. There is thus no evidence of any qualitative change in the character of the regenerated receptors. Regeneration or new production of receptors is probably a continuous physiological process irrespective of the administration of virus or RDE and the observed changes are the resultant of the two processes of destruction by RDE and of regeneration.

On the other hand there is evidence that after intensive treatment with RDE the regenerative capacity is quantitatively increased. In mice treated for fourteen days the final number of newly formed receptors was approximately 40 p.c. higher than in normal animals (hypercompensation), and the rate of production was significantly raised if regeneration after a small single dose is taken as the normal. The number of receptors dropped only slowly, and a fortnight after the last treatment with RDE was still 25 p.c. higher than in untreated control mice. These findings strongly suggest that the action of the receptor destroying enzyme is restricted to the virus receptors, the producing mechanism in the cells being unaffected at the same time. We have to postulate also—though there is nothing known at the moment about the chemistry of receptor regeneration—that the primary materials from which receptors are built up or with which biologically "destroyed" receptors are repaired, are in excess and readily available for the cell.

As the histological changes seen in the lungs after the inhalation of RDE-mist are minimal and uncharacteristic, while there is a marked difference in behaviour of the same lungs when tested for their capacity to adsorb virus, it is not justifiable to ascribe any morphological changes to the action of this enzyme. Though regeneration by its strict definition is an anatomical notion, the above described phenomena certainly belong under physiological repair processes, the more so as the time relations and the fact that hyperregeneration could be induced by strong or prolonged treatment resemble closely other banal reactions of the organism. Values calculated after the formula of Robertson and Ostwald for organic growth fit very well with the experimentally obtained regeneration curves.

## SUMMARY.

A measure of the available "receptors" for influenza virus in the mouse lung is provided by adsorption-elution experiments with a standard virus dose. The amount is approximately proportional to the time needed for 50 p.c. liberation of the virus, $T_{50}$. Where less than half the standard dose is adsorbed an approximate estimate only can be obtained.

Intranasal inoculation of the "receptor destroying enzyme" (RDE) of *V. cholerae* or inhalation of RDE mist in living mice results in reduction of available receptors in accordance with the following rules:

(a) A single effective administration is followed by progressive reduction for about 8 hours; a subsequent increase starting after 30 hours regains the normal level in approximately six days.

(b) If the action of RDE is stopped by anti-RDE serum 3 hours after administration, regeneration starts earlier (8 hours), but its rate and extent is not altered.

(c) There is no exhaustion of capacity to regenerate after 14 days' consecutive treatment with RDE—regeneration is actually more rapid than after a single effective dose.

As assayed by the routine patho-histological techniques no evident changes are produced by RDE, and no toxic effect has been observed.

It is concluded that when RDE is introduced into the mouse lung in the living animal a process of enzymic destruction of receptors by RDE is initiated. The extent of receptor destruction is only 15–20 p.c. of that obtained by the same amount of enzyme in the excised lung, and the active agent is reduced to insignificant amounts presumably by mechanical elimination within 24 hours. The resulting sequence of receptor destruction and subsequent regeneration is determined (1) by the amount of enzyme introduced and the time during which it is available for action; (2) by the rate of disappearance of RDE; and (3) by the rate of new production (or regeneration) of cell receptors.


*Acknowledgment.* It is a pleasure to thank Professor F. M. Burnet, on whose initiative this work was begun, for his advice and criticism during its completion.

## REFERENCES.


Anderson, S. G. and McCrea, J. F. (1948): Austral. J. exp. Biol., (in the press).
Burnet, F. M. (1948): (in preparation).
Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Burnet, F. M. and Stone, J. D. (1948): (in preparation).
Fazekas de St.Groth, S. (1948): Austral. J. exp. Biol., 26, p. 29.
McCrea, J. F. (1947): *Ibid.*, 25, p. 127.
Stone, J. D. (1947a): Nature 159, p. 780.
Stone, J. D. (1947b): Austral. J. exp. Biol., 25, p. 137.
Stone, J. D. (1948): *Ibid.*, 26, p. 49.
Stone, J. D. and Burnet, F. M. (1945): *Ibid.*, 23, p. 205.

# PREVENTION OF VIRUS INFECTION WITH ENZYME OF *V. CHOLERAE*.

## II. STUDIES WITH INFLUENZA VIRUS IN MICE

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In a recent communication (Stone, 1948) it was reported that chick embryos could be protected against allantoic infection with influenza virus by the administration of preparations from *V. cholerae* cultures containing the "receptor-destroying enzyme" (RDE). This enzyme which destroys the capacity of the red cell to adsorb and thereby agglutinate with viruses of the mumps-influenza group (Burnet, McCrea and Stone, 1946) also renders cells of the allantoic membrane of developing chick embryos (Stone, 1948) or of the mouse lung (Fazekas de St.Groth, 1948a) incapable of adsorbing influenza virus.

The present paper is concerned with investigations of the effect of RDE on the infection of mouse lung with influenza virus. As in chick embryos, there was considerable variation in the degree of protection afforded by RDE according to the strain of virus used. Evidence was again obtained that regeneration of the virus receptors occurs subsequent to the action of the enzyme.

A basis for the interpretation of these experiments is provided by the studies of the kinetics of receptor destruction by RDE in the mouse lung. Using a technique devised for estimating the available receptors for influenza virus from the adsorption-elution curves with a standard dose of virus, the sequence of receptor destruction and subsequent regeneration resulting from the administration of RDE has been analyzed by Fazekas de St.Groth (1948b).

## MATERIALS AND METHODS.

*Virus.* The strains of virus used in this investigation were the following:

Influenza A. PR8, Weiss,
MEL (isolated in Melbourne in 1935, mouse-adapted),
IAN (isolated in Melbourne in 1946, recently subjected to 14 serial mouse passages),
WSM (mouse adapted substrain of classical WS strain);
Influenza B. LEE;
Swine influenza (SW). Shope's strain 15.

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

Virus was prepared as infected allantoic fluid from developing chick embryos, glycerolated to a final concentration of 33 p.c. and stored at —10° C.

*Receptor destroying enzyme (RDE)* was prepared from cultures of the 4Z strain of *V. cholerae* on 1 p.c. agar plates as described previously (Burnet and Stone, 1947). The crude agar extract was heated to 55° C. at pH 6 in the presence of excess calcium chloride. This material had a titre by the standard titration method (Burnet and Stone, 1947) of 1,500–2,000 (6,000–8,000 units per ml.) In experiments of this type it is convenient to express amounts of RDE in terms of the unit defined previously (Stone, 1948). RDE was mostly used in the form of eluates prepared from the 55° C.-treated agar extract by adsorption to and elution from human red cells into buffered calcium saline (1·00 gm. $CaCl_2$, 9·00 gm. NaCl, 1·203 gm. $H_3BO_3$ and 0·052 gm. $Na_2B_4O_7.10H_2O$ per litre of distilled water, pH 7·1–7·3). Buffered calcium saline was used for the inoculation of control animals.

*Anti-RDE rabbit serum.* The properties of this serum, which was prepared by subcutaneous inoculation of RDE eluate emulsified with liquid paraffin and lanoline base, are described by Burnet (1948).

*Mouse techniques.* Mice bred in this laboratory were used at the age of 6–8 weeks. Inoculation of virus was by intranasal instillation of 0·05 or 0·1 ml. amounts under light chloroform-ether anaesthesia.

RDE was administered either by similar intranasal instillation or by exposure to a mist prepared by atomization of RDE-containing agar extract in the apparatus described by Stone and Burnet (1945). The standard period of exposure was one hour with the atomizer in operation at a pressure of 20–25 lb. per square inch for 20 p.c. of the time.

Mice were autopsied at death or on the seventh day after inoculation of virus. The extent of infection was estimated according to the convention of this laboratory from the area of lung showing consolidation, 1 = up to one-sixth of area consolidated, 2 = one-sixth to one-half, 3 = one-half to three-quarters, 4 = complete consolidation with the day of death indicated by the index number. In computing the average lesions of a group, mice dead on the seventh day scored 4·0, on the sixth day, 4·5, on the fourth or fifth day, 5·0. Intermediate readings scored as follows, 2 + = 2·5.

## Experimental.

### *Effect of RDE on Infection with Various Strains of Virus.*

In preliminary experiments with the MEL strain of influenza A virus it was found that the intranasal instillation of RDE in amounts of the order of 2,000 units per mouse together with the virus inoculum resulted in a considerable reduction in the extent of the subsequent infection. RDE was similarly effective when administered separately several hours before the virus. However, in the study of its effect on various strains of virus the simpler technique of administering it together with the virus inoculum has been adopted. Experiments dealing with the effect of the time of administration on the activity of RDE are described in a later section.

In comparative titrations of MEL diluted in buffered calcium saline and in RDE eluate containing 30,000 units per ml. the effective titre of the virus was reduced approximately 1,000 fold in the presence of RDE. Results of a typical titration are given in Table 1.

When similar experiments were carried out with the LEE strain of influenza B, the inclusion of such amounts of RDE in the inoculum brought about only a relatively slight reduction, approximately ten-fold, in the effective titre of the virus (Table 2). Results with a number of other strains of influenza tested by this technique are shown in Fig. 1. Experiments made with MEL and LEE have been included in the Figure for purposes of comparison.

TABLE 1.

*Comparative mouse titration of MEL virus in saline and in RDE.*

| Virus diluted in saline. | | | | | | | | Virus diluted in RDE.† | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution. | Lung lesions.* | | | | | | | Dilution. | Lung lesions.* | | | | | | |
| 10–6 | 2+ | 2 | 2 | 2 | 2 | · | (2·1) | 10–6 | 0 | 0 | 0 | 0 | · | · | (0·0) |
| 10–5 | 46 | 47 | 3+ | 3 | 2 | · | (3·4) | 10–5 | 0 | 0 | 0 | · | · | · | (0·0) |
| 10–4 | 46 | 46 | 46 | 47 | 47 | 3+ | | 10–4 | 3 | 2+ | 2 | 2 | 1+ | 1– | |
| | 3+ | 3 | · | · | · | · | (3·9) | | 1– | 0 | 0 | 0 | 0 | · | (1·1) |
| 10–3 | 44 | 45 | 45 | 45 | 46 | · | (4·9) | 10–3 | 3 | 2+ | 2+ | 2+ | · | · | (2·6) |

* Lung lesions are expressed as described under methods.
Value in parentheses is the arithmetic mean.
† RDE eluate containing 1,500 units in each mouse inoculum (0·05 ml.).

TABLE 2.

*Comparative mouse titration of LEE virus in saline and in RDE.*

| Virus diluted in saline. | | | | | | | | Virus diluted in RDE.† | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution. | Lung lesions. | | | | | | | Dilution. | Lung lesions. | | | | | | |
| 10–5 | 2 | 2 | 2 | 1 | · | · | (1·8) | 10–5 | 1 | 1– | 1– | 0 | 0 | · | (0·4) |
| 10–4 | 3 | 2+ | 2 | 1 | · | · | (2·1) | 10–4 | 2 | 1+ | 1+ | 1 | · | · | (1·5) |
| 10–3 | 46 | 46 | 47 | 47 | 47 | 3+ | | 10–3 | 46 | 3+ | 3 | 3 | 3 | 3 | |
| | 3 | 3 | 3 | 3 | 3 | · | (3·6) | | 2+ | 2+ | 2 | · | · | · | (3·0) |
| 10–2 | 44 | 45 | 46 | 46 | 47 | 3+ | (4·6) | 10–2 | 46 | 47 | 47 | 3+ | · | · | (4·0) |

† RDE eluate containing 1,500 units in each mouse inoculum (0·05 ml.).

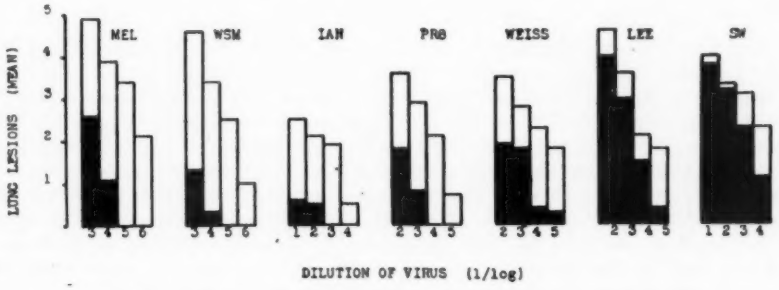


**Fig. 1.** *Effect of RDE on infection with various strains of virus.*
Clear areas represent lung lesions of control mice receiving virus in saline. Black areas represent lung lesions of mice receiving virus in RDE (1,500–2,000 units per mouse).

It will be seen that swine influenza virus resembled LEE in that the effect of RDE on infection with this strain was only slight. With the four strains of influenza A, PR8, Weiss, WSM and IAN, there was considerable reduction of infectivity, of the order of 100–1,000 fold, in the presence of RDE.

In the course of investigations, detailed below, of the conditions for optimal effectiveness of RDE it was found that greater reduction in the extent of infection with LEE virus resulted when larger amounts of RDE were administered in two doses, the first several hours before the virus and the second together with the virus.

*Neutralization of RDE with Specific Antiserum.*

The activity of RDE preparations in preventing infection in mice was neutralized by the anti-RDE rabbit serum (R228) described by Burnet (1948). Table 3 shows the results of an experiment in which 400 units of RDE given intranasally 30 minutes before an inoculum of MEL virus completely prevented the development of visible lung lesions. The admixture of an equal volume of R228 serum either undiluted or diluted 1 in 3 in saline neutralized the activity of the RDE so that there was no reduction in the extent of infection in this group as compared with the control group receiving virus alone.

TABLE 3.

*Neutralization by anti-RDE serum of the effect of RDE in preventing infection.*

| Constituents of preliminary inoculum.* | | Lung lesions. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| RDE. | Anti-RDE serum. | | | | | | | |
| Nil | Nil | 3 | 2+ | 2+ | 2 | 2 | 2 | (2·3) |
| 400 units | Nil | 0 | 0 | 0 | 0 | 0 | 0 | (0·0) |
| 400 units | 0·05 ml. | 3 | 3 | 2+ | 2 | 2 | 2 | (2·4) |
| 400 units | 0·05 ml. of 1:3 dilution | 3 | 2+ | 2+ | 2+ | 2 | . | (2·5) |

* Instilled intranasally 30 minutes before inoculum of MEL virus (1 in $10^5$ in saline).

*Absence of Lethal Effect of RDE on Virus.*

Evidence excluding the possibility of a destructive effect of RDE on the virus particle itself has already been presented (Stone, 1948) using the infectivity for chick embryos as the criterion of viability. A formal test of the effect of RDE on the mouse infectivity of the MEL strain was carried out as follows. Virus was diluted 1 in $10^3$ in buffered calcium saline and in RDE eluate (30,000 units per ml.) as used for the inoculation of mice in the experiments demonstrating prevention of infection. After incubation of these dilutions for 60 minutes at 37° C. aliquots were taken and mixed with an equal volume of normal rabbit serum (diluted 1 in 3 in saline) or of anti-RDE rabbit serum (diluted 1 in 3 in saline). The mixtures were then inoculated intranasally, 0·1 ml. per mouse. It will be seen from the results in Table 4 that there was no reduction in the infective titre of the virus after exposure to RDE for 60 minutes at 37° C.

TABLE 4.

*Mouse infectivity of MEL virus after exposure to RDE.*

| Mixture held 1 hour 37° C. | Serum added before inoculation. | Lung lesions. |
|---|---|---|
| Virus in saline | Normal rabbit | 4⁶ 4⁶ 4⁶ 4⁷ 3+ 3 (4·0) |
| Virus in RDE | Normal rabbit | 2 2 2 1+ 1 1 (1·6) |
| Virus in RDE | Anti-RDE rabbit | 4⁵ 4⁶ 4⁷ 3+ 3+ 3 (3·9) |

*Time Factor in the Administration of RDE.*

It was readily shown that RDE is effective in preventing infection in mice when administered separately from and several hours before the virus. In order to investigate the time interval giving optimal effectiveness the following experiment was performed with MEL virus. Groups of mice were given RDE by intranasal instillation at various intervals ranging from 0–24 hours before the virus inoculum. RDE was used at two levels of dosage, 400 units and 2,000 units per mouse. The results are tabulated in Table 5.

TABLE 5.

*Protection against infection with RDE given at various intervals before MEL virus.*

| Interval between RDE and virus (hours). | Lung lesions of mice receiving 400 units RDE. | Lung lesions of mice receiving 2,000 units RDE. |
|---|---|---|
| 0 | 1+ 1 1 0 0 0 (0·6) | 0 0 0 0 0 0 (0·0) |
| ½ | 0 0 0 0 0 0 (0·0) | |
| 2 | 1+ 1 0 0 · · (0·6) | 0 0 0 0 0 0 (0·0) |
| 6 | 1 1- 0 0 0 0 (0·3) | |
| 8 | 1+ 0 0 0 0 0 (0·3) | 2 0 0 0 0 0 (0·3) |
| 16 | 4 2 1 1 0 0 (1·3) | 2 1 1- 1- 0 0 (0·7) |
| 24 | 2+ 2 1+ 1+ 1- 0 (1·3) | 1 1 1- 0 0 0 (0·6) |
| Control—No RDE | 3 3 2+ 2 2 · (2·5) | |

No difference in the degree of protection afforded the mice was apparent between the groups receiving RDE together with the virus and those receiving it at an interval up to 8 hours before the virus. Given 16 or 24 hours before the virus RDE was somewhat less effective, although it still exerted a definite protective action particularly in the larger amount of 2,000 units.

If, on the other hand, the activity of RDE was arrested at the time of administration of the virus, by the admixture of anti-RDE serum to the virus inoculum, it was found that the protection afforded was roughly proportional to the time of action of the RDE for periods of short duration. RDE (400 units) was instilled intranasally into mice arranged in two

parallel series. After various intervals groups of mice in one series were inoculated with an appropriate dilution of MEL virus in normal rabbit serum (diluted 1 in 3 in saline) while those in the second series received virus diluted in anti-RDE rabbit serum (diluted 1 in 3 in saline). In general there was less protection in the groups receiving anti-RDE serum together with the virus than in the corresponding groups receiving normal serum and virus. In the latter series there was no significant variation in the protective effect which RDE exerted when given either with the virus or up to 17 hours before. This was in agreement with the experiment described above (Table 5). Given 48 hours before the virus, RDE had no protective effect in either series. In the series receiving anti-RDE serum there was some indication that the dose of RDE given 30 minutes before virus was less effective than the same amount given 6 hours before. These points are illustrated by the data in Table 6 in which the results of two experiments conducted with the same reagents at an interval of several days are combined.

TABLE 6.

*Influence of time of action of RDE on its protective effect.*

| Interval between RDE and virus (hours). | Virus administered with normal serum. | | | | | | | Virus administered with anti-RDE serum. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lung lesions. | | | | | | Reduction in mean lesions. | Lung lesions. | | | | | | | Reduction in mean lesions. |
| 0 | 1 | 1 | 0 | 0 | 0 | 0 (0·3) | 1·9 | | | | | | | | |
| ½ | 1 | 1 | 0 | 0 | 0 | 0 (0·3) | 1·9 | 2+ | 2 | 2 | 1+ | 1+ | 1 (1·8) | | 0·5 |
| 2 | 0 | 0 | 0 | 0 | 0 | . (0·0) | 2·2 | 2 | 1+ | 1 | 1 | 1 | 1 (1·3) | | 1·0 |
| 6 | 1 | 1 | 0 | 0 | 0 | 0 (0·3) | 1·9 | 1+ | 1 | 1 | 1 | 1− | . (1·0) | | 1·3 |
| Control—without RDE* | 3 | 2+ | 2+ | 2 | 1 | . (2·2) | | 3 | 3 | 2+ | 2+ | 2 | 1− (2·3) | | |
| 17 | 2 | 1 | 1− | 0 | 0 | 0 (0·7) | 1·1 | 1+ | 1+ | 1 | 1− | 1− | 0 (0·8) | | 0·8 |
| 48 | 3 | 2 | 2 | 1+ | 1 | 1 (1·8) | (0) | 3 | 2 | 2 | 2 | 2 | 1+ (2·1) | | (0) |
| Control—without RDE* | 2+ | 2 | 2 | 1+ | 1+ | 0 (1·6) | | 2 | 2 | 2 | 1 | 1 | . (1·6) | | |

* Results of two experiments are combined. Figures for the appropriate control groups are given in each case.

The relative effectiveness of a given amount of RDE administered in two doses, one several hours before the virus and the other with the virus, as compared with the same amount in a single dose with the virus, was studied in the experiment summarized in Table 7. It will be seen that with both viruses, MEL and LEE, greater protection was afforded the mice receiving RDE in two doses, the preliminary one 6 hours before the virus inoculum. In this experiment five times more RDE was required with LEE virus than with MEL to bring about a comparable reduction in infection.

TABLE 7.

*Relative effectiveness of RDE in one or two doses.*

| Virus. | Preliminary dose of RDE. | Interval. | RDE given with virus. | Lung lesions. |
|---|---|---|---|---|
| MEL | Nil | . | Nil | 3+ 2+ 2 2 1+ . (2·3) |
| MEL | Nil | . | 300 units | 3 2 1+ 1 0 0 (1·3) |
| MEL | 150 units | 6 hours | 150 units | 1+ 1 0 0 0 0 (0·4) |
| LEE | Nil | . | Nil | 3 3 2+ 1 . . (2·4) |
| LEE | Nil | . | 1,500 units | 2 1+ 1+ 1 1 1– (1·3) |
| LEE | 750 units | 6 hours | 750 units | 1+ 1 1 0 0 0 (0·6) |

In view of the above results further experiments were performed with LEE to see whether a greater reduction of infection could be demonstrated by the use of larger amounts of RDE administered in two doses. High titre RDE eluate was given in amounts of 0·05 or 0·1 ml. per mouse 18 hours or 6 hours before an inoculum containing virus plus further RDE. Under these conditions an amount of virus which caused severe infection with a proportion of specific deaths in control mice (average lesions 3·4) caused only minimal lesions (average 0·8) in mice receiving RDE in a total amount of 13,200 units (Table 8).

TABLE 8.

*Prevention of LEE infection with large amount of RDE in two doses.*

| Preliminary dose of RDE. | Interval | RDE given with virus. | Lung lesions. |
|---|---|---|---|
| Nil | . | Nil | 4[7] 4[7] 4[7] 4 3+ 3+ 3 2+ 2+ 2+ (3·4) |
| Nil | . | 2,400 units | 4[7] 3+ 3 2+ 2+ 2 (2·9) |
| 4,800 units | 6 hours | Nil | 2 2 2 2 2 . (2·0) |
| 4,800 units | 6 hours | 2,400 units | 2 2 2 1+ 1 1 (1·6) |
| 8,800 units | 6 hours | 4,400 units | 2 1 1 1– . . (1·1) |
| 4,800 units | 18 hours | Nil | 3 3 3 2+ 2+ 2 (2·7) |
| 4,800 units | 18 hours | 2,400 units | 2 2 2 1+ 1 1– (1·5) |
| 8,800 units | 18 hours | 4,400 units | 1 1 1 1 1– 0 (0·8) |

When administered after the virus inoculum RDE had relatively little effect on the development of infection. Groups of mice which had been inoculated with MEL virus 30 minutes or 2 hours previously were given RDE intranasally in a dose containing either 1,600 units or 320 units. Control mice received virus followed by an equivalent volume of saline. It will be seen from the results in Table 9 that the smaller amount of RDE which afforded practically complete protection when given simultaneously with the virus had very

little effect given 30 minutes or 2 hours after the virus. The larger dose of RDE given at either interval after virus caused some reduction in infection but was considerably less effective than the same amount given together with the virus.

TABLE 9.

*Effect of RDE on infection when given after virus (MEL).*

| Dosage of RDE. | Time of administration of RDE | Lung lesions. |
|---|---|---|
| Nil (saline control) | with virus | 3+ 3+ 3 3 3 2 (3·0) |
| Nil (saline control) | 30 minutes after virus | 3+ 3+ 3 3 3 2+ (3·1) |
| Nil (saline control) | 2 hours after virus | 3+ 3 3 3 3 2 (2·9) |
| 320 units | with virus | 1+ 0 0 0 0 0 (0·3) |
| 320 units | 30 minutes after virus | 3 2+ 2 2 2 1 (2·1) |
| 320 units | 2 hours after virus | 3 3 2+ 2+ 2 2 (2·5) |
| 1,600 units | with virus | 1− 0 0 0 0 0 (0·1) |
| 1,600 units | 30 minutes after virus | 2 2 1+ 1 1− 0 (1·2) |
| 1,600 units | 2 hours after virus | 2+ 2 2 1+ 1 1 (1·7) |

*Effect of Inhalation of RDE Mist on Virus Infection.*

Fazekas de St.Groth (1948b) has found that the lungs from mice exposed to a mist of RDE-containing fluid on several consecutive days showed extensive destruction of the virus receptors equivalent to that resulting 8–24 hours after the intranasal instillation of RDE (300 units). Consequently it was of interest to test whether mice treated in this way also showed reduced susceptibility to infection.

TABLE 10.

*Protection against infection by inhalation of RDE mist.*

| Virus inoculum. | Time of inoculation after exposure to RDE. | Lung lesions of mice pre-treated with RDE mist. | Lung lesions of control mice. |
|---|---|---|---|
| MEL 10–6 | 1½ hours | 1− 0 0 0 0 0 (0·1) | 2 2 1+ 1 1− 0 (1·2) |
| MEL 10–6 | 19 hours | 2 1+ 1 1 1 . (1·3) | 1+ 1+ 1 1 . . (1·1) |
| MEL 10–5 | 1½ hours | 1+ 1+ 1+ 1 0 0 (0·9) | 3 2+ 2 2 2 1+ (2·2) |
| MEL 10–5 | 19 hours | 2+ 2+ 2 2 2 2 (2·2) | 3 2+ 2 2 . . (2·4) |

Mice were exposed to a mist prepared by atomization of RDE-containing agar extract for periods of one hour on six consecutive days. Half of the mice so treated were inoculated with MEL virus at an interval of 1½ hours after completion of the last mist treatment while the remainder received an equivalent dose of virus after an interval of 19 hours. As indicated by the results in Table 10 this treatment afforded considerable protection against

infection with virus given within 1½ hours but had no effect against virus given after the longer interval. The extent of protection in the former groups was somewhat less than that resulting from the intranasal instillation of 400 units of RDE.

The possibility that the daily exposure of mice to RDE mist might enhance the protection afforded by a single intranasal dose of RDE by controlling receptor regeneration was investigated as follows. Mice were exposed to RDE mist for one hour on the four days following inoculation of virus together with RDE, commencing 24 hours after the administration of virus. With both MEL and LEE viruses there was no difference in the extent of infection in the mice treated in this way as compared with the animals receiving RDE in a single dose only.

*Regeneration of Virus Receptors.*

It is evident that the effect of RDE in modifying the susceptibility of the lung to virus infection is not a lasting one but that there is a fairly rapid recovery of susceptibility just as there is regeneration of the virus receptors as detected by the excised lung technique. In experiments of the present type an absolute measure of the time required for regeneration is impracticable owing to the probability of the survival of the virus inoculum in the lung for a considerable period of time. Some indication of the regeneration time can be gained, however, from the experiments described above. For example, it was found that RDE instilled intranasally 48 hours before the virus inoculum had no detectable effect on infection (Table 6).

In an experiment designed to show the phenomenon of regeneration more directly, mice were given 400 units of RDE intranasally followed after 8 hours by anti-RDE serum, then after a further interval of 16 hours or longer by an inoculum of MEL virus. In no case was there evidence of any reduction in the extent of infection.

## DISCUSSION.

Previous experiments (Stone, 1948) on the activity of RDE in preventing virus infection in chick embryos were interpreted in terms of the hypothesis that the susceptibility of the host cell to infection with influenza virus is dependent, in the first instance at least, on the presence on its surface of specific receptor areas to which virus is adsorbed. The activity of RDE in preventing infection is thus attributed to the "destruction" of these cell receptors so that virus cannot be adsorbed. Further support to this hypothesis is lent by the findings reported in this paper as viewed in the light of the experiments of Fazekas de St.Groth (1948b) analyzing the changes in the level of available receptors for influenza virus in the mouse lung following administration of RDE.

From the latter work it was concluded that the intranasal instillation of RDE or the inhalation of RDE mist results in a progressive reduction in the number of available receptors for the first 6 to 8 hours. The number remains at a minimal level until the twenty-fourth hour approximately, and then rises again, regaining the normal level in about 6 days. If the activity of RDE is arrested by the instillation of anti-RDE serum, regeneration of receptors is demonstrable, at approximately the same rate, from the eighth hour so that in mice receiving anti-RDE serum 8 or more hours after RDE it commences at the time of

administration of the antiserum. Evidence was obtained that RDE was eliminated from the lungs of living mice within 24 hours.

In considering the results of infectivity experiments it must be borne in mind that the virus inoculum probably remains viable in the lung for some considerable time even though it is unable to infect immediately. Hence it is not only the level of receptors existing in the lung at the time of inoculation of virus which determines whether or not infection will take place but also the changes in this level, due on the one hand to the further action of RDE, on the other to regeneration, which occur within the survival time of the virus inoculum.

An examination of the experimental findings, reported above, on this basis reveals a close correlation between the susceptibility of the mouse lung to virus infection and the level of available receptors following the administration of RDE. Thus the maximal effect of RDE in preventing infection results from its intranasal instillation up to 8 hours before virus. The lesser effect when it is given at longer intervals before the virus can be attributed to regeneration of receptors within the survival time of the virus inoculum.

Although the minimal level of receptors as measurable by the excised lung technique is not reached until 6 to 8 hours after the intranasal instillation of RDE in amounts of this order (400 units), RDE given simultaneously with virus appeared as effective as the same amount given 6 to 8 hours before virus. The explanation of this finding becomes apparent if it is assumed that RDE, in addition to destroying unoccupied receptors, can actually remove adsorbed virus from the cell. This has been shown to be the case in the excised mouse lung. The progressive reduction in the effectiveness of RDE given 30 minutes or 2 hours after virus would indicate a fairly rapid penetration of virus into the cell or at least a modification of the initial form of attachment so that it cannot be displaced by RDE.

One might expect that the optimal effectiveness of RDE in preventing virus infection would be obtained with repeated doses at intervals such that the level of available receptors was maintained at a minimal value throughout the time of exposure to virus. Daily exposure of mice to RDE mist maintained the number of virus receptors in the lungs at the lowest level measurable by the excised lung technique. However, one would expect that this level would be considerably above the threshold level for infection, as the amount of virus used as indicator in the excised lung technique was of the order of $10^5$ times that used in the infection experiments. Hence, the finding, described above, that the repeated exposure of mice to RDE mist at intervals of 24 hours failed to enhance the effect of RDE intranasally instilled with the virus inoculum, may well be due to regeneration of receptors under these conditions to a level enabling initiation of infection. The time interval between treatments giving best results would probably be in the region of 8 hours, as a single dose of RDE results in a progressive reduction in the number of available receptors over this period. It

was found experimentally that the administration of a standard amount of RDE in two doses, one 6 hours before virus and the other simultaneously with virus, was more effective in preventing infection than a single dose with the virus. Application of this principle in the case of the relatively insusceptible strain, LEE, resulted in almost complete protection of mice from infection with a dose of virus producing severe infection or death in control animals.

In view of the findings of Taylor (1941) that intranasal instillation of sterile fluid enhances the development of lung infection in mice inoculated one or more days previously with influenza virus, no attempt was made to administer further doses of RDE by intranasal instillation following the inoculation of virus. Actually the most convenient method would probably be that of exposure to RDE mist at intervals of, say, 12 hours. The volume of fluid which reaches the lung during the inhalation of mist is relatively small—in the case of mice exposed for one hour it was calculated to be approximately 0·004 ml. (Fazekas de St.Groth, 1948b). Hence it would be necessary to use fluids containing a high titre of RDE in the preparation of the mist.

The relative insusceptibility of the LEE strain to the effect of RDE in preventing infection in mice is in agreement with results of similar experiments in chick embryos (Stone, 1948). It was not possible to test other strains of influenza B as we have been unsuccessful in adapting them to mice.

SUMMARY.

Intranasal instillation of the receptor-destroying enzyme of *V. cholerae* simultaneously with the virus inoculum protected mice against infection with the viruses of influenza A and B and of swine influenza. The extent of the protection afforded by a standard dose of enzyme varied considerably with the different strains of virus tested. In most cases protection against infection with 100 to 1,000 infective doses was demonstrable.

The receptor-destroying enzyme was effective in preventing infection when administered up to 24 hours before the virus. Its effectiveness was considerably reduced when it was instilled after the virus. The treatment affording the greatest protection appeared to be the administration of a double dose of enzyme, the first 6 to 18 hours before and the second together with the virus.

Mice were similarly protected against infection following inhalation of enzyme in the form of a mist on several successive days.

It was shown that the enzyme has no direct action on the virus, its effect being wholly on the host cell.

The activity of the enzyme in preventing infection was neutralized by specific rabbit antiserum.

These results are discussed in terms of the available virus receptors in the mouse lung at the time of exposure to the virus as conditioned by their enzymatic destruction and subsequent regeneration.

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REFERENCES.

Burnet, F. M. (1948): In preparation.
Burnet, F. M., McCrea, J. F. and Stone, J. D. (1946): Brit. J. exp. Path., 27, p. 228.
Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Fazekas de St.Groth, S. (1948a): *Ibid.*, 26, p. 29.
Fazekas de St.Groth, S. (1948b): *Ibid.*, 26, p. 271.
Stone, J. D. (1948): *Ibid.*, 26, p. 49.
Stone, J. D. and Burnet, F. M. (1945): *Ibid.*, 23, p. 205.
Taylor, R. M. (1941): J. exp. Med., 73, p. 43.

# THE BEHAVIOUR OF *HAEMOPHILUS PERTUSSIS* IN CASEIN HYDROLYSATE BROTH

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The introduction of fluid media of simple composition by Hornibrook (1939) was a major advance in the production of pertussis vaccine. Their use facilitates large scale work and eliminates the danger of sensitization to animal proteins derived from the Bordet Gengou medium.

Wilson (1945) working with casein hydrolysate-starch broth noticed considerable variation between different batches of the same medium, the variation concerning final yield of bacteria, type of dispersion and even change from Phase I. Large inocula were required in order to obtain satisfactory results.

The inadequacy of Hornibrook's (1939) original medium was pointed out by Cohen and Wheeler (1946) who modified it by decreasing the salt content and increasing the starch.

The discovery of pertussis haemagglutinin by Keogh, North and Warburton (1947) provided a powerful new tool for investigating the properties of *Haemophilus pertussis*, and in particular characters of its fluid culture supernates. Using a medium essentially identical with Cohen and Wheeler's (1946) considerable variations were observed in regard to rate and yield of bacterial growth and haemagglutinin production. An investigation was therefore undertaken with the object of studying the characters of these cultures and elucidating the causes of the variations.

## METHODS.

*Strain.* The strain used was strain "P 71" isolated from a typical case of whooping cough in September 1946 and maintained in the lyophilized state. It is in Phase I and is virulent for mice.

*Medium.* Cohen and Wheeler's (1946) modification of Hornibrook's medium was used.

Preparation of the casein hydrolysate: Casein was precipitated from fresh skim milk with weak acetic acid. The precipitate was collected, dissolved in dilute ammonia, re-precipitated with weak acetic acid, and this process repeated once. The final precipitate was dried with subsequent lots of cold alcohol and defatted by successive treatments with acetone, hot alcohol and several lots of each of the following solvents: benzene, diethyl ether, petroleum ether and again diethyl ether. Evaporation of the solvents after extraction yielded decreasing quantities of fatty material as the extraction progressed, but the last lot of ether still left a small lipoid residue. The last traces of ether were driven off in the incubator and the dry

material was ground and passed through a fine sieve. Hydrolysis of this casein was carried out according to a modification of Mueller and Johnson's (1941) method. The casein was refluxed for 6 hours with 10 N HCl, using 250 ml. acid for 50 gm. of casein. The acid and water were evaporated, as far as possible, from a boiling water bath, *in vacuo*. The residue was taken up in hot water, cooled down and neutralized with a measured amount of 15 p.c. NaOH. The mixture was clarified by boiling with "Supercell" and "Nuchar" and filtering hot through paper on a Buchner funnel; the process of clarification was repeated once. Total solids were determined and the material steamed and stored in the cold.

No sodium chloride was added to the medium, as there was enough in the casein hydrolysate to give a final concentration of about 0·3 p.c., as indicated by the amount of NaOH required for neutralization. Otherwise the medium was made up as described by Cohen and Wheeler (1946) and included 0·15 p.c. soluble starch (Merck's).

The medium thus prepared was sterilized in the autoclave and dispensed aseptically in 5 ml. lots into cylindrical 50 ml. bottles.

*Inocula.* The growth was scraped off the surface of 24-hour Bordet Gengou slopes and suspended in the sterile culture broth to a turbidity corresponding approximately to 2–3,000 million organisms per ml. The suspension was filtered aseptically through glass wool. Eight serial tenfold dilutions in the broth were prepared aseptically, from this initial inoculum, using a fresh pipette for each dilution. Live counts on these inocula were determined by spreading 0·1 ml. lots of the three highest dilutions on triplicate Bordet Gengou plates.

Batches of bottles containing 5 ml. broth were inoculated from each inoculum dilution, adding 0·5 ml. seed dilution to each bottle.

*Incubation* was done at 36° C. Uninoculated "blanks" were incubated alongside the cultures.

*Observation of progress.* Duplicate bottles from each inoculum group were removed after 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 days' incubation. The purity of these samples was checked by smear and Gram stain. Turbidity determinations were carried out on the Klett-Summerson photometer; the cultures were then spun clear in the angle centrifuge. The supernates were separated and their pH values determined on the glass electrode. The bacteria were resuspended in saline to the original volume and haemagglutinin titrations were carried out on the supernatants and on the bacterial suspensions.

The "blanks" were used to follow the changes of turbidity and of pH in the sterile medium on incubation.

The amount of evaporation was determined by measuring the volume of fluid in all remaining bottles on the twelfth day.

*Haemagglutination tests.* Ten-drop volumes of culture supernates or bacterial suspensions were diluted serially two-fold in Wassermann tubes with neutralized M/200 cysteine hydrochloride in normal saline. One drop of 5 p.c. washed human "O" cells was added to each tube. The tubes were shaken and read after settling at 37° C. or at room temperature, using sedimentation pattern as the indicator of the end-point. The titre was taken as the reciprocal of the highest dilution giving complete agglutination; partial agglutinations were not recorded.

## EXPERIMENTAL RESULTS.

The results are summarized in Figs. 1 and 2 and in Table 1. Allowances have been made for the turbidity changes in the "blanks" and for the progressive evaporation. The colony counts showed that the cultures containing the

lowest inocula had been seeded with approximately 12 live bacteria per ml.; accordingly, the cultures receiving the largest inocula had been sown with $1{\cdot}2\times10^8$ live organisms per ml. culture.

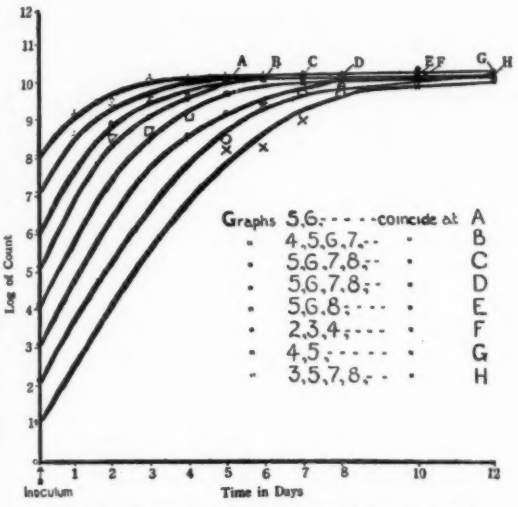


**Fig. 1.** Curves 1–8 indicate the rate of growth from the eight inoculum groups. For each curve, the starting point is the number of live bacteria per ml. culture (12 for Curve 1, and increasing tenfold for each successive curve). The initial values were determined by plate counts; all subsequent ones by turbidity measurements.

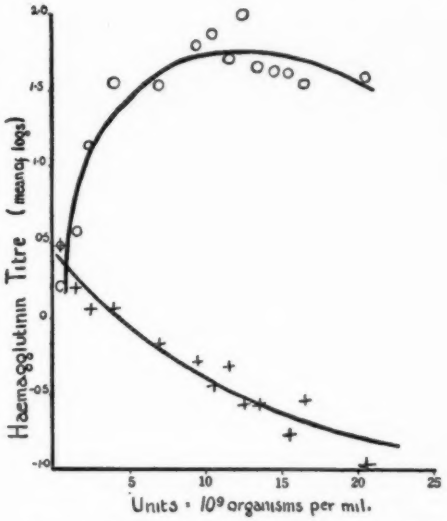


**Fig. 2.** The upper curve indicates the progress of haemagglutinin titres of the supernates; the lower one, the haemagglutinating power of the organisms. For explanation, see text.

The *growth curves* (Fig. 1) indicate that *H. pertussis* is a slowly growing organism producing only about 3 generations per day. In this experiment growth occurred from quite small inocula—about 12 live organisms per ml.—and neither the rate of multiplication nor the final yield of bacteria were influenced by the size of the inoculum. This may be taken to indicate that the medium was highly favourable. The final yields of bacteria ranged between 10,000 million and 20,000 million organisms per ml., figures comparable with those of Wilson (1945).

As the growth proceeded, aggregates large enough to be seen by the naked eye appeared and soon afterwards the faint straw-coloured supernate assumed a yellow tint which gradually deepened on further incubation. The granularity may have been due to increased electrolyte concentration associated with evaporation. The yellow colour was probably caused by autolysis; at the same time the cultures become increasingly viscous, and loss of bacterial outline and disintegration of the organisms could be seen in the stained smears.

The growth curves follow a practically linear course at first, then commence to flatten out at a concentration of about 1,000 million organisms per ml. This means that 90 to 95 p.c. of organisms in a fully developed culture were produced during a period of reduced activity. This fact may have some bearing on the haemagglutinin content of the organisms.

The *pH values* are given in Table 1. In the actively growing cultures the pH values steadily rose from 7 to 8–8·4, then slowly fell to about 7·9 during the ageing of the culture.

TABLE 1.

*pH values of cultures.*

| Age in Days. | Number of live organisms in inoculum per ml. culture. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $1·2\times10$ | $1·2\times10^2$ | $1·2\times10^3$ | $1·2\times10^4$ | $1·2\times10^5$ | $1·2\times10^6$ | $1·2\times10^7$ | $1·2\times10^8$ |
| 1 | 7 | 6·9 | 6·9 | 6·9 | 6·9 | 6·9 | 7 | 7·3 |
| 2 | 6·8 | 6·8 | 6·8 | 6·8 | 6·9 | 7 | 7·3 | 7·7 |
| 3 | 6·7 | 6·7 | 6·7 | 6·8 | 6·9 | 7·3 | 7·5 | 8·2 |
| 4 | 6·6 | 6·7 | 6·8 | 7·1 | 7·7 | 8·2 | 8·3 | 8·3 |
| 5 | 6·7 | 6·9 | 7·1 | 7·8 | 8·4 | 8·4 | 8·3 | 8·3 |
| 6 | 6·8 | 7·3 | 7·6 | 8·4 | 8·3 | 8·3 | 8·3 | 8·2 |
| 7 | 7 | 8 | 8·2 | 8·2 | 8·2 | 8·2 | 8·1 | 8·1 |
| 8 | 7·9 | 7·5 | 8·2 | 8·3 | 8·1 | 8·1 | 8·1 | 8 |
| 10 | 7·8 | 8·1 | 8·1 | 8·1 | 8·1 | 7·9 | 7·9 | 7·9 |
| 12 | 8 | 8·1 | 8 | 8·1 | 8·2 | 8·1 | 7·9 | 8·1 |

No visible growth was present in the cultures corresponding to the readings in the upper left-hand portion of the Table, above the line.

The pH of the uninoculated "blanks" gradually fell to 6·5–6·6 during the first 5 or 6 days, then remained stationary at that value. The early fall of pH in the cultures seeded with small inocula was therefore non-specific. It is of interest to note, however, that these cultures were apparently in no way affected by the low pH and kept on multiplying at the same rate as the others. The ability of the organisms to grow at pH 6·3–6·5 has since been observed on other occasions.

*Haemagglutinin Content of Organisms and Supernates.*

The use of doubling dilutions in the haemagglutinin titrations, the difficulty of reading end points accurately and possibly also some variation in the erythrocytes resulted in considerable variations in the haemagglutinin values. In all inoculum groups, however, definite trends could be observed. The haemagglutinin content of the bacteria fell with the increase of the total count; the titres of the supernates rose first and then slightly declined. In order to summarize the information, the titres were grouped together according to the counts of the samples, irrespective of actual age, or size of inoculum. The titres

of the supernates were expressed as described above, and the titres in the organisms as the number of haemagglutinating units per 1,000 million organisms. Means of the logarithms of these figures were calculated for each group and the results plotted against the counts (Fig. 2).

The rise in the haemagglutinin content of the supernates coincides with the rise in pH. At a count of about 10,000 million organisms per ml. the curve reaches its peak, then gradually falls during the ageing of the culture. The highest actual titres read in this experiment were about 200.

The haemagglutinin content of the bacteria falls at a slightly decreasing rate. The opposite direction of the two curves suggests liberation of haemagglutinin from the bacteria into the supernate by the young organisms. It is possible that bacterial generations in the flattening portion of the growth curve contain less haemagglutinin than those in the earlier phase. Furthermore, some destruction of the haemagglutinin probably occurs in the culture. The amount of haemagglutinin necessary to agglutinate a given quantity of red cells may be less when it is in solution than when it is fixed on the surface of the bacteria. For these reasons, exact quantitative relationships between bound and free haemagglutinin cannot be established from the data.

*Some Factors Bearing on the Suitability of the Medium.*

It was observed that batches of medium differing only in the source of casein hydrolysate varied greatly in their suitability to grow *H. pertussis.*

Fats are common impurities in commercial casein. On drastic acid hydrolysis these most probably split, yielding fatty acids. Butter fat is rich in unsaturated fatty acids, some of which have been shown to be toxic to the tubercle bacillus and to a micrococcus species by Dubos (1947) who also found that this toxic effect is neutralizable by bovine albumin, and that the addition of bovine albumin and "Tween 80," an ester of oleic acid, enhance the growth of the tubercle bacillus.

Starch is known to combine with higher fatty acids. It forms a precipitate with oleic acid (Wilson, Schoch and Hudson, 1943).

It seemed possible that fatty impurities in the casein were responsible for the variation in the growing quality of the different batches of the medium, and that if the effect of starch were neutralization of fatty impurities, replacement of the starch by albumin might be possible or even an advantage. The latter possibility was tested by experiments, one of which is described below.

The casein hydrolysate was prepared from a sample of moderately pure commercial casein. The medium was made up without starch. Aqueous solutions of 1·5 p.c. strength were made up of six different starches and two albumin preparations. The starch solutions were autoclaved and the albumin solutions sterilized by filtration. One of the albumin samples was prepared from ox serum by alcohol precipitation in the cold. The other sample was the dialyzed and lyophilized residue of horse serum, following removal of the globulins by ammonium sulphate precipitation.

The medium was dispensed in 1 ml. lots into eight rows of eight $\frac{3}{4} \times 5$ in. test tubes. 0·1 ml. lots of the various starch and albumin solutions were added to tubes of the different rows. The inocula were made up, filtered and diluted as described in the previous section, using the basic medium without starch or albumin.

0·1 ml. of each inoculum dilution was added to one tube in each row. Live counts were determined on Bordet Gengou plates as described in the previous section.

The cultures were observed after 1, 2, 3, 4, 6, 7, 8 and 9 days' incubation. Those showing growth were then checked for purity by smear.

Results with these media are given in Table 2. It appears that the rate of multiplication, the ability to support growth from small inocula and, to some extent, the final yield of bacterial mass are functions of the suitability of the medium. Slight differences were found between the various samples of starch tested, but the batch of medium containing the crude horse-albumin was far superior to all the others.

TABLE 2.

| Ingredient. | Number of live organisms in inoculum per tube. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $3 \times 10^7$ | $3 \times 10^6$ | $3 \times 10^5$ | $3 \times 10^4$ | $3 \times 10^3$ | $3 \times 10^2$ | 30 | 3 |
| Soluble starch (Merck's) | 1<br>+++4 | 3<br>+++8 | 8<br>+9 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| BDH fat-free and vitamin-free starch | 1<br>+++3 | 1<br>+++6 | 2<br>+++8 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| BDH maize starch | 1<br>+++4 | 2<br>+++4 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| BDH potato starch | 1<br>+++3 | 1<br>+++4 | 3<br>+++6 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| BDH rice starch | 1<br>+++4 | 1<br>+++4 | 2<br>+++4 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| BDH wheat starch | 1<br>+++6 | 1<br>+++6 | 3<br>+++7 | 4<br>±4 | –<br>– | –<br>– | –<br>– | –<br>– |
| Alcohol precipitated bovine albumin | 1<br>++2 | 1<br>++3 | 2<br>++3 | –<br>– | –<br>– | –<br>– | –<br>– | –<br>– |
| Crude horse-albumin | 1<br>++2 | 1<br>++2 | 2<br>+++6 | 2<br>+++6 | 3<br>+++6 | 4<br>+++7 | –<br>– | –<br>– |

*Key.* For every tube showing growth, the top figure indicates the day when growth became perceptible to the naked eye. Underneath this are indicated the final degree of turbidity and the day when this was reached.

± signifies perceptible growth, + signifies slight growth, ++ signifies moderate growth, and +++ signifies heavy growth.

The effects of raising the percentage of starch were also investigated, and the optimum concentration was found to be 1·5–2 p.c. Our routine medium now contains starch in this proportion.

The recent paper by Pollock (1947) on this subject reached us as this report was being written. Our results are in general agreement with his. It does not appear certain, however, that the action of lipoid materials in the medium is wholly toxic. We found that if the fats be extracted from the broth by shaking it with successive lots of ether prior to autoclaving, its growing qualities deteriorate rather than improve. Similar results are obtained if the medium is made with casein hydrolysate which has been subjected to extraction with ether in the dry state in the Soxhlet apparatus. It seems possible that lipid combined with certain substances is a growth factor for the organisms, whereas the same lipid in the free state is toxic. According to Dubos (1947) this is the case for the tubercle bacillus. Although the evidence is suggestive, we have no direct proof of this hypothesis as regards *H. pertussis*.

## DISCUSSION.

It appears from the foregoing study that the quality of casein hydrolysate broth, in regard to its ability to support the growth of *H. pertussis*, depends to a considerable extent on the casein hydrolysate and, given constant conditions of hydrolysis, probably on the sample of casein. The substances responsible for this property of the casein hydrolysate are probably lipoid in nature and their toxic effect is offset by suitably prepared albumin and to a lesser extent by starch.

A good medium will support growth from small inocula, at a rate of multiplication of about 3 generations a day till a total count of about 1,000 million organisms per ml. is reached, then at a gradually falling rate to a final yield of 10–20,000 million organisms per ml.

The cultures exhibit a characteristic pH rise to values between 8 and 8·4, then a gradual fall.

Under adverse conditions large inocula are required to initiate growth. The rate of multiplication is lower and the yield of bacterial mass sometimes less.

The haemagglutinin content of the bacteria decreases with the ageing of the culture. The haemagglutinating power of the supernatant rises sharply during the period of maximum multiplication, then slightly declines.

## SUMMARY.

The characteristics of cultures of *H. pertussis* in casein hydrolysate broth are discussed, including rate of growth, growth from small inocula, changes of pH and formation of haemagglutinin.

The effects of the quality and composition of the medium on the cultures are described and a tentative hypothesis is put forward to explain the findings.

The quality of indifferent batches of medium may be improved by raising the starch content to 1·5–2 p.c.

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REFERENCES.

Cohen, S. M. and Wheeler, M. W. (1946): Amer. J. Pub. Health, 36, p. 371.
Dubos, R. J. (1947): J. exp. Med., 85, p. 9.
Hornibrook, J. W. (1939): Pub. Health Rep., 54, p. 1,847.
Keogh, E. V., North, E. A. and Warburton, M. F. (1947): Nature, 160, p. 63.
Mueller, J. H. and Johnson, E. R. (1941): J. Immunol., 40, p. 33.
Pollock, M. R. (1947): Brit. J. exp. Path., 28, p. 295.
Wilson, E. J., Schoch, T. J. and Hudson, C. S. (1943): J. Amer. chem. Soc., 65, p. 1,380.
Wilson, R. J. (1945): Canad. J. Pub. Health, 36, p. 321.

# THE INHIBITION OF CATALASE BY ASCORBIC ACID

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*(Accepted for publication, 16th March, 1948.)*

Reducing substances such as ascorbic acid, cysteine, glutathione (cf. Stern, 1932; Waldschmidt-Leitz, 1933; Marks, 1936) and BAL (2:3-dimercaptopropanol) (Webb and van Heyningen, 1947) are well-known catalase inhibitors. Keilin and Hartree (1938) have shown that carbon monoxide, although without effect on pure catalase, strongly increases the inhibition of catalase by cysteine and glutathione. We find the same to hold true for BAL (Lemberg and Foulkes, 1948). This shows that in the presence of the above reducing substances and hydrogen peroxide, catalase is reduced to the ferrous state.

Lemberg and Legge (1943) found that ascorbic acid inhibits catalase by accelerating the destruction of the enzyme by hydrogen peroxide. Furthermore, it was shown that bile pigment haemoprotein is formed from protohaem catalase by the action of large amounts of hydrogen peroxide. As only ferrous haem compounds are oxidized by hydrogen peroxide to bile pigment compounds, it appeared that in the presence of hydrogen peroxide and ascorbic acid, catalase is reduced to the ferrous state, just as in the presence of sulphhydryl compounds. Carbon monoxide, then, should also increase the inhibitory effect of ascorbic acid on catalase. The experiments reported in the present paper show, however, that this is not the case. Hence the present theory of the mode of ascorbic acid inhibition of catalase requires modification.

## Ascorbic Acid Inhibition of Catalase.

### *Materials and Methods.*

Various horse liver catalase preparations were used for this investigation. They were obtained by the method of Zeile and Hellström (1930) as modified by Lemberg and Legge (1943), except for the use of ammonia instead of alkaline phosphate for elution from alumina This obviates the need for dialysis before further purification. All preparations had Kat-f. values of 20,000–30,000 as measured in the usual way.

Catalase activities were measured in Warburg manometers shaken at 120 strokes per minute. The enzyme was added from the side arm to the mixture of substrate and inhibitor in the main compartment. Molarities of catalase are expressed in terms of haematin concen-

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tration. In all experiments there always was excess hydrogen peroxide, i.e. enzyme concentration was limiting.

Carbon monoxide was prepared by the action of sulphuric on formic acid. It was stored over water. Before use it was passed through a strongly alkaline solution of sodium dithionite ($Na_2S_2O_4$) containing anthraquinone sulphonate, and through a charcoal trap. Gas mixtures were prepared over water. Copper was used in the form of a standard solution of $CuSO_4.7H_2O$ in dilute acid. Copper was estimated by the method of Mann and Keilin (1938) with diethyldithiocarbamate. The concentration of the yellow copper complex was determined by means of a Spekker photoelectric absorptiometer, using a violet filter. An accuracy of $\pm 0{\cdot}4\mu g.$ copper was obtained with standard solutions containing 2–20$\mu g.$ copper. Ascorbic acid and diethyldithiocarbamate were crystalline commercial products.

*The Effect of Carbon Monoxide and of Copper on the Ascorbic Acid Inhibition.*

Figure 1 shows that carbon monoxide, instead of increasing the inhibition of catalase by ascorbic acid, partly reverses it.

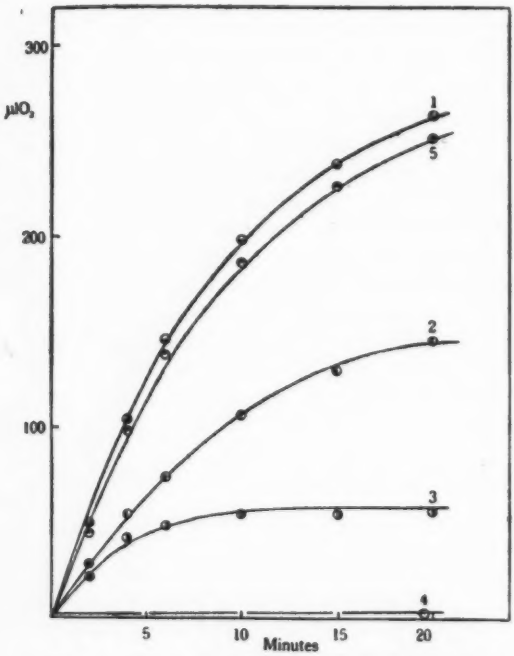


**Fig. 1.** Influence of CO on ascorbic acid-catalase. Catalase $8 \times 10^{-10}$ M, $H_2O_2$ $10^{-2}$ M, $PO_4$ $3 \times 10^{-4}$ M. Ascorbic acid and dehydroascorbic acid, $10^{-4}$ M. Total volume 3 ml. Temperature 26° C. pH 6·8. (1) Control in air or CO; (2) Ascorbic acid in CO; (3) Ascorbic acid in air; (4) Control in air without catalase; (5) Dehydroascorbic acid in air.

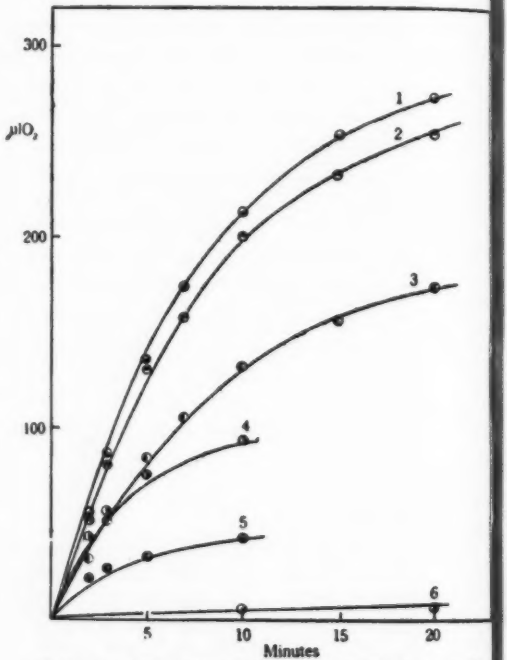


**Fig. 2.** Influence of copper. Conditions as for Fig. 1, Cu $3{\cdot}7 \times 10^{-5}$ M. (1) Control in air; (2) Control + copper in air; (3) Ascorbic acid in CO; (4) Ascorbic acid in air; (5) Ascorbic acid plus copper in air; (6) Ascorbic acid + copper, but no catalase, in air.

The results shown are typical of many experiments although the actual degree of reversal varied within rather wide limits. Table 1 shows that the carbon monoxide effect is specific, i.e. it is not due to decrease of the partial pressure of oxygen.

TABLE 1.

*Influence of $pO_2$ and various gases on ascorbic acid inhibition.*

Experiments A, B, C are not comparable to each other. They differ in the concentration of catalase and ascorbic acid used. Oxygen output in $\mu$l.

| Gas. | Ascorbic acid. | Experiment A. | Experiment B. | Experiment C. |
|---|---|---|---|---|
| CO | + | 122 | . | . |
| Air | + | 38 | 50 | 48 |
| $N_2$ | + | 39 | 51 | . |
| $H_2$ | + | . | 52 | . |
| CO 80 p.c., $O_2$ 20 p.c. | + | . | . | 274 |
| Air | — | . | 205 | . |

This reversal of the ascorbic acid inhibition by carbon monoxide suggested that ionic copper might be concerned in the interaction of ascorbic acid and catalase.

Fig. 2 shows that ionic copper significantly increases the ascorbic acid inhibition of catalase. Copper at the concentration used, caused little inhibition by itself. It is only at higher concentrations, e.g. $2 \times 10^{-4}$ M, that copper has any appreciable inhibitory action.

The action of carbon monoxide in reversing ascorbic acid inhibition would appear to be due to the binding of copper. This is supported by the results described in Table 2. Here the copper was bound by chelation with diethyldithiocarbamate. Under these conditions carbon monoxide had no effect on the activity of catalase in the presence of ascorbic acid.

TABLE 2.

*CO effect in presence of diethyldithiocarbamate.*

Diethyldithiocarbamate $3 \times 10^{-3}$ M, ascorbic acid $3{\cdot}6 \times 10^{-4}$ M, catalase $4 \times 10^{-3}$ M, $H_2O_2$, $2 \times 10^{-2}$ M; pH 6·8. Temperature 37° C.

| Gas. | $\mu$l. $O_2$ output/20 minutes. |
|---|---|
| CO | 96 |
| | 96 |
| Air | 89 |
| | 100 |

If in the presence of ascorbic acid, the effect of carbon monoxide were twofold; namely: (a) combination with ferrous catalase, counterbalanced by (b) binding of copper, carbon monoxide should have had an inhibitory effect in the experiments described in Table 2.

In a concentration of $3 \times 10^{-3}$ M, diethyldithiocarbamate itself depressed catalatic activity. If used in a concentration of $3 \times 10^{-4}$ M, however, it has no inhibitory action on catalase by itself and acts like carbon monoxide in partly reversing ascorbic acid inhibition.

All these experiments suggest that copper is essential for the ascorbic acid inhibition of catalase. The stock solutions of catalase contained an average of 40$\mu$g. p.c. copper. As the catalase was diluted about $10^5$ times for experimental purposes, this can hardly be a serious source of contamination. It was found, on the other hand, that appreciable amounts of copper were introduced in the phosphate buffer. The final concentration of copper in the above experiments was of the order of $10^{-7}$ M.

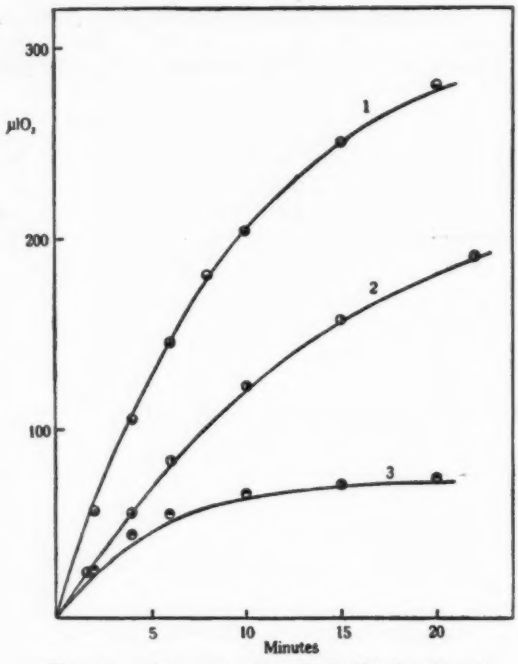


**Fig. 3.** Reversal by diethyldithiocarbamate. Catalase $1{\cdot}1 \times 10^{-9}$ M; $H_2O_2$ $1{\cdot}4 \times 10^{-2}$ M; $PO_4$ $9{\cdot}5 \times 10^{-4}$ M; ascorbic acid $2{\cdot}9 \times 10^{-4}$ M; diethyldithiocarbamate $3 \times 10^{-4}$ M. Temperature 26° C. Total volume 2·1 ml. pH 6·8. (1) Control; (2) Diethyldithiocarbamate plus ascorbic acid; (3) Ascorbic acid.

Copper-free phosphate was then prepared by extraction with 8-hydroxy-quinoline and chloroform. As control, some phosphate was treated with chloroform only. Double glass distilled water was used for solutions and dilutions. In this way, the concentration of copper was reduced below the level of sensitivity ($4 \times 10^{-8}$ M) of the method of estimation used. Under these conditions we found a decreased ascorbic acid inhibition of catalase.

TABLE 3.

*Ascorbic acid inhibition in solutions with reduced copper content.*

Ascorbic acid $3 \times 10^{-4}$ M, phosphate $6{\cdot}6 \times 10^{-4}$ M, $H_2O_2$ $1{\cdot}5 \times 10^{-2}$ M, catalase $8 \times 10^{-10}$ M. pH 6·8. Temperature 26° C.

| Solution. | Concentration of copper. | $\mu$l. $O_2$/20 minutes. |
|---|---|---|
| A | $10^{-7}$ M | 203 |
| A + ascorbic acid | | 67 |
| B | below | 207 |
| B + ascorbic acid | $4 \times 10^{-8}$ M | 114 |

### The Nature of the Ascorbic Acid Inhibition.

Copper may interact (a) with ascorbic acid, (b) with the catalase protein or (c) with a compound of catalase and ascorbic acid.

(a) Copper catalyses the autoxidation of ascorbic acid according to the following equation: $C_6H_8O_6+O_2=C_6H_6O_6+H_2O_2$.

One might assume that copper, by increasing the oxygen consumption by ascorbic acid, would diminish the overall oxygen output, and that carbon monoxide, by binding copper, would reverse this effect. The oxygen uptake, according to the above equation, corresponding to the total ascorbic acid present, is, however, quantitatively insufficient to account for the reversal of the ascorbic acid inhibition by, for example, carbon monoxide. Nor can the effect of copper be explained by assuming some copper-catalysed destruction of ascorbic acid, for in this case carbon monoxide, by binding copper and thus protecting the ascorbic acid, should increase the inhibition of catalase.

The products of the copper-catalysed autoxidation of ascorbic acid are also not responsible for the effect of copper. There is always present an excess of hydrogen peroxide, and dehydroascorbic acid, prepared by oxidation of ascorbic acid with the theoretical amount of iodine in a well-buffered solution at pH 6·8, does not inhibit catalase (see Fig. 1, curve 5).

(b) Direct combination of copper with the protein of catalase as an explanation for the copper effect is excluded because, at the small concentration of copper used, there is little inhibition by copper itself (see Fig. 2).

TABLE 4.

*Reversal by phosphate.*

Ascorbic acid $4{\cdot}4 \times 10^{-4}$ M, $H_2O_2$ $1{\cdot}4 \times 10^{-2}$ M, catalase $8 \times 10^{-10}$ M. pH 6·8. Temperature 26° C.

| Concentration of phosphate. | $\mu$l. $O_2$ output/20 minutes. | |
|---|---|---|
| | With ascorbic. | Without ascorbic. |
| M/105 | 133 | 116 |
| M/2,100 | 54 | 185 |

Nor is the action of ascorbic acid plus copper similar to that found by Mapson (1946) with urease,[2] since ascorbic acid must combine with catalase to exert its inhibition. We have previously described a ferric compound of catalase and ascorbic acid (Lemberg and Foulkes, 1948). Its dissociation constant, measured spectrophotometrically in ·035 M phosphate pH 7·0 at 565 m$\mu$, is

[2] Here ascorbic acid acts simply by reducing cupric to cuprous ion; the latter is the true inhibitor.

$4{\cdot}3\times10^{-4}$. The combination of ascorbic acid with catalase is further indicated by the fact that high concentrations of phosphate also reverse the action of ascorbic acid on catalase.

In view of the work of Agner and Theorell (1946) on anion-catalase combination the effect of phosphate can be explained as a competition between phosphate and ascorbate ions.[3]

(c) A possible explanation for the combined rôle of ascorbic acid and copper would be that the ascorbic acid in the catalase iron-ascorbate complex reduces an S–S group in the catalase protein. The sulphhydryl group formed, which would normally be reoxidized by hydrogen peroxide, could be immobilized by combination with copper. Against this hypothesis speaks the fact that even in the presence of ascorbic acid, iodoacetic acid has no effect on catalase.

TABLE 5.

*Effect of iodoacetate plus ascorbate.*

Ascorbic acid $1{\cdot}5\times10^{-4}$ M; iodoacetate $10^{-2}$ M, catalase $2\times10^{-9}$ M; $H_2O_2$ $10^{-2}$ M. Temperature 37° C. pH 6·8.

| | $O_2$ output in $\mu$l./10 minutes. |
|---|---|
| Catalase | 200 |
| Catalase + ascorbate | 131 |
| Catalase + iodoacetate | 199 |
| Catalase + ascorbate + iodoacetate | 140 |

It is of interest in this regard that the evidence for the presence of –SH or –S–S– groups in the catalase protein is conflicting (Barron and Singer, 1945; Cook and co-workers, 1946; see also Carruthers, 1947).

## SUMMARY.

The ascorbic acid inhibition of catalase is partly reversed by carbon monoxide, by low concentrations of diethyldithiocarbamate, and by high concentrations of phosphate. The inhibition is increased by the addition of copper, and is decreased in solutions with reduced copper content.

It is concluded that the presence of copper is required for ascorbic acid inhibition of catalase.

There is no evidence for the formation of ferrous catalase in the presence of ascorbic acid and low concentrations of hydrogen peroxide.

Possible mechanisms of the inhibition are discussed.

[3] (Annotation during proof-reading.) Meanwhile, we have found, however, that formate which combines more strongly with catalase iron than phosphate, does not reverse the ascorbic acid inhibition. The explanation of the phosphate effect given above, is thus incorrect.

## REFERENCES.

Agner, K. and Theorell, H. (1946): Arch. Biochem., 10, p. 321.
Barron, E. S. G. and Singer, T. B. (1945): J. biol. Chem., 157, p. 221.
Carruthers, C. (1947): *Ibid.*, 171, p. 641.
Cook, E. S. and co-workers (1946): *Ibid.*, 162, p. 43.
Keilin, D. and Hartree, E. F. (1938): Proc. Roy. Soc. Lond. B., 124, p. 397.
Lemberg, R. and Legge, J. W. (1943): Biochem. J., 37, p. 117.
Lemberg, R. and Foulkes, E. C. (1948): Nature, 161, p. 131.
Mann, T. and Keilin, D. (1938): Proc. Roy. Soc. Lond. B., 126, p. 303.
Mapson, L. (1946): Biochem. J., 40, Proc., p. 5.
Marks, G. W. (1936): J. biol. chem. 115, p. 299.
Stern, K. G. (1932): Z. physiol. Chem., 209, p. 176.
Waldschmidt-Leitz, E. (1933): *Ibid.*, 214, p. 75.
Webb, E. C. and van Heyningen, R. (1947): Biochem. J., 41, p. 74.
Zeile, K. and Hellström, H. (1930): Z. physiol. Chem., 194, p. 171.

# THE HAEMAGGLUTININ OF *HAEMOPHILUS PERTUSSIS*.

## I. HAEMAGGLUTININ AS A PROTECTIVE ANTIGEN IN EXPERIMENTAL MURINE PERTUSSIS.

by E. V. KEOGH AND E. A. NORTH

(From the Commonwealth Serum Laboratories, Melbourne).

*(Accepted for publication, 16th March, 1948.)*

In the course of unsuccessful attempts to isolate a substance assumed to be responsible for the haemolysis produced by *Haemophilus pertussis* on Bordet Gengou medium, it was noticed that erythrocytes of various species were agglutinated by saline suspensions of the organisms, and by the cell-free supernatant fluids of centrifuged broth cultures. Further investigation showed that this was not an example of so-called non-specific pan agglutination, since the haemagglutinating property of such suspensions and supernates was neutralizable by pertussis antisera. Our conclusions concerning the correlation between the haemagglutinin content of strains of *H. pertussis*, their virulence for mice, and the protective potency of vaccines prepared from them have been reported in brief (Keogh, North and Warburton, 1947). This paper records the experimental data on which these conclusions were based, and some additional observations.

## MATERIALS AND METHODS.

*Strains.* Phase I strains isolated from patients with typical whooping cough, lyophilized immediately after isolation. In comparative experiments, strains were employed which have been maintained at these laboratories for months or years on Bordet Gengou slopes.

*Media.* (a) Bordet Gengou, containing 30 p.c. defibrinated horse blood. (b) Casein digest broth as originally devised by Hornibrook (1939) and modified by Cohen and Wheeler (1946). Growth was initiated with moderately large inocula from overnight cultures on Bordet Gengou slopes, from 100–1,000 million organisms per 10 ml. of medium. The broth was dispensed in shallow layers in Erlenmeyer flasks or in Roux bottles (10 ml. in 100 ml. flasks, 200 ml. in Roux bottles). A single batch of "Difco" Casamino Acids was used in preparation of the lots of media employed in these experiments.

*Preparation of Haemagglutinating Suspensions and Supernates.*

(1) *Suspensions.* The growth from overnight cultures on Bordet Gengou slopes at 37° C. was emulsified in normal saline containing 0·01 p.c. cysteine hydrochloride, neutralized with NaOH, in a concentration of 10,000 million organisms per ml.

(2) *Supernates.* The growth in casein digest broth was harvested from the third to the fifth day of incubation at 37° C. when titrations of uncentrifuged samples showed that an adequate or maximum titre of haemagglutinin had been reached. The cultures were centrifuged for 30–60 minutes in an angle centrifuge until all the organisms had been deposited. The supernatant fluids were carefully removed and, when necessary, re-centrifuged. Formalin

to a concentration of 0·3 p.c. was added to the clear supernates, which were then lyophilized, unless required for immediate use.

*Titration of Haemagglutinin.*

Graded dilutions, usually doubling, of suspensions or supernates were prepared by the drop method in normal saline, containing 0·01 p.c. cysteine hydrochloride, neutralized with NaOH. To ten drops of each dilution one drop of 5 p.c. saline suspension of washed fowl or human erythrocytes was added, and the tubes allowed to stand on the bench, or at 37° C., for from a half to two hours, depending on the type of erythrocytes used. End-points were read according to the patterns of the sedimented cells, as in virus haemagglutination techniques.

*Titration of Anti-Haemagglutinin in Immune Sera.*

Graded dilutions of sera were titrated against a fixed dose of soluble haemagglutinin, usually 8 minimal complete haemagglutinating doses. One drop of 5 p.c. washed erythrocytes was added to 5 drops of the serum dilutions immediately prior to or after the addition of 5 drops of haemagglutinin. In all such tests, a human convalescent serum (AC8) of high anti-haemagglutinin titre was used as a reference standard. This serum was arbitrarily regarded as containing 100 anti-haemagglutinin units per ml. and the strengths of other sera were expressed in such units, by comparison of their titres with that of the reference serum. The reference serum neutralizes 8 haemagglutinating doses in a dilution of 1:160, using this technique.

Since neutralization of haemagglutinin by immune sera obeys the law of multiple proportions, titrations can also be carried out by using a fixed dose of serum and varying the dose of haemagglutinin. For titrations of maximum accuracy it would obviously be advisable to use as large a dose of haemagglutinin as practicable. For the purpose of these experiments, doubling dilutions of serum were employed, and comparisons of the different patterns of the sedimented cells used to obtain slightly more accuracy in the serum titrations.

*Method of Infection of Mice.*

Mice were infected intranasally by the method of Burnet and Timmins (1937). This method has been employed at these laboratories for some ten years with satisfactory and reproducible results. (North, Keogh, Anderson & Williams, 1939; North, Anderson and Graydon, 1941; North, 1946).

## RESULTS.

### *The Appearance of Extra-Cellular Haemagglutinin in Fluid Cultures.*

The times at which soluble haemagglutinin first appears in the fluid, and at which it reaches a maximum, are dependent on the size of the inoculum and the suitability of the batch of medium. The total yield is also dependent on the strain of *H. pertussis* employed. The factors in the medium affecting haemagglutinin production are discussed by Fisher (1948a). If 10 ml. volumes of a suitable batch of casein digest broth are inoculated with ten million organisms of a freshly isolated strain of *H. pertussis*, haemagglutinin usually appears in solution after 48 hours' incubation and the maximum titre is reached between the third and fifth day. The growth rate of the bacteria and the titres of haemagglutinin in one experiment are shown in Table 1.

TABLE 1.

*Production of pertussis haemagglutinin in fluid cultures.*

| Days of incubation. | Titre: Supernate. | Titre: Resuspended organisms. | Bacterial density millions per ml. |
|---|---|---|---|
| 1 | 1, 2 | <1, 1 | 330, 430 |
| 2 | 80, 80 | 2, 4 | 1,300, 1,400 |
| 3 | 80, 160 | 8, 8 | 3,600, 3,900 |
| 4 | 1,280, 2,560 | 32, 64 | 5,000, 6,000 |
| 5 | 1,280, 2,560 | 32, 32 | 5,000, 5,500 |

Casein digest broth (10 ml. lots) inoculated with *H. pertussis* (250 million organisms). Duplicate samples removed daily, spun on angle centrifuge, cell-free supernates removed, deposited organisms resuspended in casein digest broth; haemagglutinin titrations performed immediately.

The haemagglutinin titres of the supernates were exceptionally high in this instance; usually values lay between 1:16 and 1:128, and averaged 1:64.

*Haemagglutinin Content and Yields of Different Strains.*

We had access to numerous strains of *H. pertussis* which have been maintained for months or years, in the Vaccine Department of these laboratories, by frequent passage on Bordet Gengou medium. These strains are to some extent selected, as it is the practice to discard those which lose virulence for mice by the intranasal route, or which fail to grow luxuriantly on Bordet Gengou medium after several passages. All these strains are in Phase 1 as judged by colonial characters, by agglutination to titre by Phase 1 sera, and by production of Phase 1 agglutinating antibody in rabbits. A selection was tested, in comparison with fresh isolations, for haemagglutinin content, by estimating the titre of suspensions from cultures on solid media, and for production of soluble haemagglutinin in liquid culture. The results are shown in Table 2.

It will be seen that the strains vary considerably in haemagglutinin content, and that the haemagglutinin content is correlated with the ability to produce soluble haemagglutinin in fluid culture. While there is a general tendency for the older strains to possess or produce little haemagglutinin, some strains have retained these properties to a considerable extent for many years, while some recently isolated strains have lost them. Insufficient freshly isolated strains have been examined to determine whether they vary greatly in haemagglutinin content or production on isolation. Such differences as have been observed could be related to variation in different batches of solid or liquid media. Tests of the strains recorded in Table 2 were carried out simultaneously with the same lots of media. We have, however, noted reduction of haemagglutinin content within a few weeks in some freshly isolated strains maintained on Bordet Gengou medium.

TABLE 2.

*Haemagglutinin content and production in relation to age of* H. pertussis *strains.*

| Strain. | Period since isolation. | Titre: Bacteria. | Titre: Supernates. |
|---|---|---|---|
| 12 | 11 years | 4 | <1 |
| 13 | 11 years | <1 | <1 |
| 15 | 11 years | 8 | 1 |
| 27 | 11 years | 4 | <1 |
| 34 | 10 years | 32 | 4 |
| 36 | 10 years | 4 | <1 |
| 40 | 9 years | 2 | <1 |
| 41 | 9 years | 8 | 1 |
| 43 | 8 years | 2 | 1 |
| 45 | 8 years | 8 | <1 |
| 50 | 7 years | 64 | 1 |
| 59 | 7 years | 2 | 2 |
| 60 | 3 years | 4 | <1 |
| 61 | 2 years | 1,024 | 256 |
| 62 | 2 years | <1 | 1 |
| 63 | 3 months | 64 | 256 |
| 64 | 3 months | 256 | 64 |
| 65 | 2 months | 256 | 512 |
| 66 | 2 months | 128 | 128 |

Bacterial suspension of density 10,000 organisms per ml. from 18 hour Bordet-Gengou slopes.

The supernatant fluids, obtained in angle centrifuge, of three-day broth cultures inoculated with 250 million organisms.

Titres are the reciprocal of the highest dilution in which definite haemagglutination was observed.

TABLE 3.

*Relation of haemagglutinin content to virulence of* H. pertussis *strains.*

| Strain. | Haemagglutinin titre of bacterial suspension. | Infecting dose of bacteria (millions). | Day of death. | Survivors. |
|---|---|---|---|---|
| P13 | <1 | 50 | | 10/10 |
| P60–8 | 4 | 50 | 5, 5, 6, 8, 12, | 5/10 |
| P45 | 8 | 50 | 5, 5, 5, 5, 10, 12, | 4/10 |
| P34 | 32 | 50 | 3, 5, 5, 6, 6, 7, 7, | 3/10 |
| P65 | 256 | 50 | 1, 1, 1, 1, 2, 2, 2, 2, 2, 3 | 0/10 |
| P65 | | 17 | 2, 2, 2, 2, 3, 3, 3, 3, 4, 7, | 0/10 |
| P65 | | 5 | 4, 5, 5, 7, 7, 7, 10, 11 | 2/10 |

Haemagglutinin titre is the reciprocal of the dilution of a bacterial suspension, density 10,000 million organisms per ml., which produced definite haemagglutination.

*Virulence of Strains in Relation to Their Haemagglutinin Content.*

Phase 1 strains were selected, of varying haemagglutinin content, for intranasal infection of mice. The results are shown in Table 3.

It will be noted that the strain with highest haemagglutinin content shows maximal virulence. Those with moderate haemagglutinin content are less virulent, while the strain lacking haemagglutinin is practically avirulent. These findings have been confirmed on a number of other strains on various occasions.

The results are strictly applicable only to virulence tests in mice in which the intranasal route is used. Endotoxin is involved in the mortality in mice infected intraperitoneally (Anderson and North, 1943) and factors other than haemagglutinin probably influence the death date in intracerebral infections (Fisher, 1948b).

*The Relation of Haemagglutinin Content and Virulence of Strains to the Antigenic Potency of Vaccines.*

It has been shown that the virulence of strains for mice, using the intranasal route for infection, is closely related to their haemagglutinin content. An expected corollary is that vaccines prepared from strains rich in haemagglutinin would be the immunizing agents of choice. Several experiments with a number of strains have been in accord with this assumption. A protocol of one such trial is shown in Table 4.

TABLE 4.

*Haemagglutinin content, mouse virulence, and immunizing potency of two strains of* H. pertussis.

| Strain. | Haemagglutinin titre of bacterial suspensions. | Non-immunized mice. | | Immunized mice. | |
|---|---|---|---|---|---|
| | | Day of death. | Survivors. | Day of death. | Survivors. |
| P15 | 16 | 2, 3, 3, 3, 4, 6, | 4/10 | 2, 3, 3 | 4/7 |
| P61 | 1,024 | 1, 1, 1, 1, 1, 2, 2, 2, 2, 3, | 0/10 | | 7/7 |
| | | | Controls | 1 1 1 2 2 2 3 | 0/7 |

Non-immunized mice infected intranasally with 50 million organisms of respective strains.

Immunized mice received 2 doses of 50 million formolized organisms of respective strains and were challenged with 50 million organisms of a different recently isolated strain 10 days after the second immunizing dose.

*Protection Against Intranasal Infection by Immunizing with Soluble Haemagglutinin.*

Mice are protected as efficiently against intranasal infection by prior immunization with soluble haemagglutinin as with a vaccine freshly prepared from a fully virulent strain. In such experiments we have commonly used the

supernatant fluids of broth cultures which undoubtedly contain other soluble products of the organisms in addition to haemagglutinin. Several experiments using broth supernates have shown that their immunizing potency is related to their haemagglutinin content. One experiment comparing the protection conferred by a single dose of haemagglutinin in the form of a culture supernate given ten days prior to infection with that given by a standard vaccine is shown in Table 5.

TABLE 5.

| Infecting dose of organisms. | Immunized mice. Haemagglutinin. Day of death. | Haemagglutinin. Survivors. | Immunized mice. Vaccine. Day of death. | Vaccine. Survivors. | Controls. Day of death. | Controls. Survivors. |
|---|---|---|---|---|---|---|
| $50 \times 10^6$ | 2, 2, 2, 3, 3, 3, 3, 3, 4, 4, 4, 5, 8, 9, | 1/16 | 2, 3, 4, 5, 7, 7 | 4/10 | 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, | 0/10 |
| $17 \times 10^6$ | 2, 2, 3, 3, 3, 4, 4, 8, 8, | 7/16 | 5, 5, 6, 7, 7, 9, 10 | 3/10 | 2, 2, 2, 3, 3, 3, 5, 5, 10 | 1/10 |
| $5 \times 10^6$ | 9, 14 | 13/15 | 4, 4, 5, 5, 5, 6 | 4/10 | 3, 4, 6, 6, 6, 7, 7, 11, 15, 16 | 1/10 |

Mice immunized with a single intraperitoneal dose of (a) 0·25 ml. of cell-free supernate, haemagglutinin titre 1:64; (b) 250 million formol-killed organisms of same strain as used for production of haemagglutinin.

Mice challenged 10 days after dose with same strain.

In experiments of which the details are not recorded in this paper, we have protected mice by immunization with washed erythrocytes to which the haemagglutinin in broth supernates has been adsorbed.

It will be noted that the mice immunized with haemagglutinin showed only slight evidence of protection against the highest challenging infective dose. That there was, however, some protection against this dose is evidenced by the longer survival times. Protection against the two smaller challenge doses was more pronounced than in the mice immunized with vaccine. The total survivors in the haemagglutinin immunized mice in all groups were 21 of 47 challenged; in the vaccine group 11 of 30 survived. Only 2 of 30 control mice survived. The difference in favour of the haemagglutinin immunized group as against the vaccine group is not statistically significant, but the difference between each group of immunized mice and the control group is highly significant. In several trials it has been noted that the slope of the mortality curve in vaccine-immunized mice is very flat (*vide* North, Anderson and Graydon, 1941), whereas

a steep mortality curve is noted with increase of the challenging dose in mice, immunized actively with haemagglutinin as in this experiment, or passively with immune sera of high anti-haemagglutinin titre (North *et al.*, 1939).

*The Relation of Protective Power of Antisera to Their Anti-Haemagglutinin Content.*

As stated previously, we have employed a human convalescent serum (AC8) as a reference standard for many years, because we have noted that the serum given to mice intraperitoneally was highly protective against intranasal infection with *H. pertussis*. This serum has a low Phase I agglutinin titre. Various rabbit sera of high Phase 1 agglutinin titre failed to protect passively under similar conditions (North *et al.*, 1939). It was found early in the present investigations that this serum had a high content of anti-haemagglutinin, and that the protective potency of sera given intraperitoneally against intranasal infection was directly related to their anti-haemagglutinin titres. An illustrative experiment is shown in Table 6. The challenge dose in this experiment was deliberately adjusted to cause about 50 p.c. mortality, so that the effects of minimal amounts of protective antibody might be detected.

TABLE 6.

*Relation of anti-haemagglutinin content of sera to protective potency.*

| Serum. | Source. | Titre bacterial agglutinin | Anti-haemagglutinin units per ml. | Dose of anti-haemagglutinin (units). | Deaths in challenged mice. |
|---|---|---|---|---|---|
| AC8 | Human | 10 | 100 (standard) | 20·0<br>6·6 | 2/10<br>3/10 |
| P64 | Rabbit | 20,000 | 400 | 80·0<br>26·6<br>8·0<br>2·7 | 0/10<br>1/10<br>3/10<br>5/10 |
| P62 | Rabbit | 20,000 | 50 | 10·0<br>3·3<br>1·0<br>0·3 | 1/10<br>6/10<br>5/10<br>5/10 |
| Nil | — | — | — | — (controls) | 5/10 |

0·2 ml. of graded dilutions of sera in saline given intraperitoneally four hours prior to intranasal infection of mice with 20 million *H. pertussis* (strain P61). Mice observed for 7 days.

Of the mice given 10 or more anti-haemagglutinin units, 4 of 40 died. Of the mice given $<10$ and $>5$ anti-haemagglutinin units, 6 of 20 died. Of the mice given $<5$ anti-haemagglutinin units, 21 of 40 died. The differences between these groups are statistically significant.

The two rabbit sera used both had the high Phase 1 agglutinin titres of 1:20,000. They differed considerably in anti-haemagglutinin content. The reference serum AC8 has a high anti-haemagglutinin content, and a negligible Phase 1 agglutinin titre. The data demonstrate clearly that such protection as is conferred against intranasal infection by pertussis antisera given intraperitoneally is a direct function of their anti-haemagglutinin content, and bears no relation to their Phase 1 agglutinin titres. It should be stated that the reference serum AC8 contains no detectable anti-endotoxin (Anderson and North, 1943). Before concluding that the protective value of immune sera in experimental murine pertussis is solely a function of their power to neutralize haemagglutinin, it should be remembered that, though passive protection may not be afforded by high titre Phase 1 agglutinating sera given intraperitoneally, the same sera show marked protective potency if given intranasally before, with, or shortly after the infecting dose; though a marked prozone is usual (North, 1946).

## SUMMARY.

Saline suspensions of *H. pertussis,* and cell-free supernates of fluid cultures, agglutinate erythrocytes of various species.

The virulence of strains of *H. pertussis,* and the protective potency in experimental murine pertussis of vaccines prepared from them, is related to their haemagglutinin content.

The potency of pertussis antisera, when administered intraperitoneally, in protecting mice against intranasal infection is a function of their anti-haemagglutinin content.

## REFERENCES.

Anderson, G. A. and North, E. A. (1943): Austral. J. exp. Biol., 21, p. 1.
Burnet, F. M. and Timmins, C. (1937): Brit. J. exp. Path., 18, p. 83.
Cohen, S. M. and Wheeler, M. M. (1946): Amer. J. Publ. Hlth., 36, p. 371.
Fisher, S. (1948a): Austral. J. exp. Biol., 26, p. 299.
Fisher, S. (1948b): (unpublished observations).
Hornibrook, J. W. (1939): U.S. Publ. Hlth. Reports, 54, p. 1,847.
Keogh, E. V., North, E. A. and Warburton, F. M. (1947): Nature, 160, p. 63.
North, E. A., Keogh, E. V., Anderson, G. A. and Williams, S. W. (1939): Austral. J. exp. Biol., 17, p. 275.
North, E. A., Anderson, G. A. and Graydon, J. J. (1941): Med. J. Austral., 2, p. 589.
North, E. A. (1946): Austral. J. exp. Biol., 24. p. 253.

# THE INFLUENCE OF PAST EXPERIENCE ON THE LIBERATION OF HISTAMINE AND ITS IMMUNOLOGICAL SIGNIFICANCE

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It has already been shown that there is a natural variation in the histamine content of the guinea-pig lung with age. At birth the content is very high, thereafter there is a rapid fall followed by a gradual rise from 8–40 weeks of age (Trethewie, 1947a). Burnet (1940) suggested the possibility that the higher reactivity in the young adult age group might be associated either with a higher histamine content of certain tissues or with an increased ease of liberation in response to the stimulus of micro-organismal attack. Since there was a considerable increase in the extreme variation of the histamine content of the lung with age, it was thought worth while to determine if outside factors influenced the histamine content of the lung or the ease of liberation of histamine. This has been investigated in this paper. Any change in reactivity to another injurious agent after repeated contact with a former agent would suggest that there is a non-specific effect which may have immunological significance.

## METHODS.

*Selection of Animals.*

Fifty-six animals were obtained from a constant breeding source which had not been altered for a considerable time. They were placed in a pen of their own. After randomization, the animals to be injected with peptone were tagged in the left ear and the control in the right ear. At the age of two weeks the young pigs were left to look after themselves and at this time, injections of Tyrode for the control and peptone for the treated group were commenced. Hereafter the latter will be designated "peptone" animals and the former called control. Seven injections were made at intervals of three weeks subcutaneously over the abdomen. Four to six weeks after the last injection the lungs of the survivals of Nos. 1–24 were perfused with Tyrode. The $l.d._{50}$ for histamine was determined on the remainder. The pigs were weighed regularly.

*Lung Perfusion.*

The lung perfusions were carried out by the method of Feldberg and Kellaway (1937), Tyrode being used. The left lung was removed and the right lung remained. The perfusion rate was 0·9–1·6 ml./min. after the blood was washed out and the ventilation rate was

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11/min. Trypsin (Difco) was injected into the arterial cannula (10 mg. in 0·5 ml.). This was allowed to act for 1½ minutes while the flow was interrupted. The perfusion was then continued for 40 minutes during which time the perfusion fluid was collected and then boiled.

The entire left lung was squeezed repeatedly between filter papers to remove all excess fluid and weighed. The right lung after perfusion was excised, placed in a small funnel and gently squeezed and allowed to drain for 20 minutes. This constituted drainage fluid. This fluid was also boiled. The right lung was squeezed similarly to the left between filter papers and then weighed, cut finely, extracted with Tyrode and the volume made up 1:5. This was also boiled. The left lung was similarly extracted.

*Histamine Assay.*

Histamine was estimated as histamine acid-phosphate on the isolated jejunum of the guinea-pig suspended in Tyrode.

*Lethal Dose of Histamine.*

Some animals received injections into the jugular vein directly and the remaining animals were injected subcutaneously over the abdomen. The concentration of histamine acid-phosphate was 1 mg./ml. for venous injection and 10 mg./ml. for subcutaneous injection.

## EXPERIMENTAL.

1. *Injection of Animals.*

Fifty-six animals were selected at birth and numbered one to fifty-six in order of birth. The animals were all obtained over a period of 18 days from a constant breeding source. They were randomized into two equal groups after six had died. At the age of two weeks each pig in one group was injected subcutaneously over the abdomen with 5 ml./kg. Tyrode. The pigs in the second group were injected similarly with 2 gm./kg. peptone dissolved in Tyrode. At intervals of three weeks six similar additional injections were made in all animals, the last five injections being 4 ml./kg. At the age of 25 weeks, perfusion experiments were performed. In the meantime pigs numbered 23, 33 and 48 died (Tyrode injected) and pigs numbered 15, 25, 30, 34, 35, 37, 39, and 46 (peptone injected) died. Peptone may have been responsible for the greater number of deaths in the latter group but the difference is not statistically significant. The weights of the Tyrode-injected pigs were, in general, greater than of the peptone-injected pigs, the averages being 791 and 760 gm. respectively.

2. *Perfusion Experiments.*

At the age of 25 weeks the lungs of the guinea-pigs were perfused and injected with trypsin, one experiment being performed daily until fifteen were completed. The perfusions and histamine estimations were carried out by one of us who was not aware of the group to which each pig belonged, the injections having been performed by the other. A technician, who also was unaware of the group to which each animal belonged, supervised the ventilation of the lungs so that it was maintained approximately constant from experiment to experiment. In this way it was hoped to minimize the personal equation. The ventilation was considerable, so that as much as possible of the fluid in the tissue spaces was collected during the period of perfusion. Finally, the drainage fluid was also obtained after cutting the lung down.

TABLE 1.

*Details of experiments recorded in this paper.*

| Pig No. | Wt. of pig gm. | Wt. lung before (L) gm. | Wt. lung after (R) gm. | Lung content of histamine before (L) γ/gm. | Lung content of histamine after (R) γ/gm. | Output of histamine in perfused fluid | Volume of perfused fluid ml. | Output of histamine in drainage fluid. | Volume of drainage fluid ml. | Histamine output (γ) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control 2 | 680 | 2·25 | 3·76 | 17 | 15 | 1/17 mill. | 58 | 1/3 mill. | 2·8 | 4·3 |
| Control 5 | 645 | 1·96 | 3·6 | 38 | 25 | 1/35 ,, | 59 | 1/900,000 | 4·2 | 6·4 |
| "Peptone" 6 | 675 | 2·4 | 2·9 | 19 | 17 | 1/10 ,, | 55 | 1/1·9 mill. | 2·0 | 6·6 |
| Control 7 | 735 | 1·75 | 2·5 | 39 | 30 | 1/53 ,, | 47 | 1/670,000 | 1·3 | 2·8 |
| "Peptone" 8 | 575 | 1·8 | 2·3 | 100 | 89 | 1/1·25 ,, | 34 | 1/100,000 | 1·1 | 38·2 |
| Control 9 | 610 | 1·74 | 2·32 | 50 | 34 | 1/3·5 ,, | 55 | 1/2·5 mill. | 1·7 | 16·3 |
| Control 10 | 650 | 1·9 | 2·5 | 54 | 49 | 1/7 ,, | 62 | 1/1·25 mill. | 2·1 | 10·6 |
| "Peptone" 11 | 750 | 2·14 | 2·75 | 29 | 21 | 1/3·6 ,, | 56 | 1/400,000 | 0·8 | 17·6 |
| "Peptone" 12 | 730 | 1·84 | 2·80 | 57 | 33 | 1/5 ,, | 56 | 1/600,000 | 1·5 | 13·7 |
| Control 13 | 685 | 1·77 | 2·71 | 91 | 60 | 1/4·6 ,, | 53 | 1/200,000 | 1·7 | 20 |
| Control 17 | 700 | 1·39 | 2·25 | 65 | 43 | 1/16 ,, | 57 | 1/700,000 | 1·6 | 5·9 |
| Control 20 | 670 | 1·44 | 2·17 | 24 | 18 | 1/9 ,, | 55 | 1/1·1 mill. | 1·2 | 7·2 |
| Control 21 | 635 | 1·49 | 1·60 | 42 | 29 | 1/25 ,, | 52 | 1/6 mill. | 0·7 | 2·2 |
| Control 22 | 775 | 1·52 | 2·00 | 41 | 35 | 1/35 ,, | 55 | 1/400,000 | 1·0 | 4·1 |
| "Peptone" 24 | 650 | 1·54 | 2·32 | 80 | 54 | 1/1·25 ,, | 57 | 1/1·25 mill. | 1·4 | 46·7 |

The details in one experiment (Control 10) were as follows: The lungs of the pig (injected with Tyrode on seven occasions) weighing 650 gm. were perfused with Tyrode and the left lung weighing 1·9 gm. was removed. Perfusion of the right lung was continued, ventilated at 11 per minute and 10 mg. trypsin dissolved in 0·5 ml. Tyrode were injected into the pulmonary artery cannula and allowed to act for 90 seconds. After the flow was recommenced 62 ml. of perfusion fluid were collected over a period of 40 minutes and contained 1:7 mill. histamine. The uninjected left lung contained 54γ histamine/gm. and the right lung, which weighed 2·5 gm. after squeezing between filter papers, contained 49γ histamine/gm. after perfusion. Drainage fluid (2·1 ml.) contained 1:1·25 mill. histamine. Thus the total output of histamine was 10·6γ.

TABLE 2.

*Output of histamine in perfusion and drainage fluid.*

(a) Control.

| Pig. No. | Perfused sample. Volume ml. | Perfused sample. Histamine content γ. | Drainage fluid. Volume ml. | Drainage fluid. Histamine content γ. | Total output γ. |
|---|---|---|---|---|---|
| 2 | 58 | 3·4 | 2·8 | 0·9 | 4·3 |
| 5 | 59 | 1·7 | 4·2 | 4·7 | 6·4 |
| 7 | 47 | 0·9 | 1·3 | 1·9 | 2·8 |
| 9 | 55 | 15·6 | 1·7 | 0·7 | 16·3 |
| 10 | 62 | 8·9 | 2·1 | 1·7 | 10·6 |
| 13 | 53 | 11·5 | 1·7 | 8·5 | 20·0 |
| 17 | 57 | 3·6 | 1·6 | 2·3 | 5·9 |
| 20 | 55 | 6·1 | 1·2 | 1·1 | 7·2 |
| 21 | 52 | 2·1 | 0·7 | 0·12 | 2·2 |
| 22 | 55 | 1·6 | 1·0 | 2·5 | 4·1 |
| Av. | 55·3 | 5·5 | 1·8 | 2·4 | 8 |

(b) "Peptone."

| Pig. No. | Perfused sample. Volume ml. | Perfused sample. Histamine content γ. | Drainage fluid. Volume ml. | Drainage fluid. Histamine content γ. | Total output γ. |
|---|---|---|---|---|---|
| 6 | 55 | 5·5 | 2·0 | 1·1 | 6·6 |
| 8 | 34 | 27·2 | 1·1 | 11·0 | 38·2 |
| 11 | 56 | 15·6 | 0·8 | 2·0 | 17·6 |
| 12 | 56 | 11·2 | 1·5 | 2·5 | 13·7 |
| 24 | 57 | 45·6 | 1·4 | 1·1 | 46·7 |
| Av. | 51·6 | 21 | 1·4 | 3·5 | 24·6 |

The findings in a "peptone" experiment were as follows: Pig 24, weighing 650 gm., received previously seven injections of peptone. The lungs were perfused and the left lung, weighing 1·54 gm., was removed. The right lung was perfused and ventilated at 11 per minute and injected with 10 mg. trypsin which was allowed to act for 90 seconds. After the flow was recommenced 57 ml. of perfusate were collected over a period of 40 minutes and contained 1:1·25 mill. histamine. The left (uninjected) lung contained 80γ histamine/gm.

and the right lung, which weighed 2·32 gm. after perfusion and squeezing, contained 54γ histamine/gm. The drainage fluid (1·4 ml.) obtained after cutting the lung down, contained 1:1·25 mill. histamine. Thus the total output of histamine was 46·7γ.

The findings in all these cases are recorded in Table 1. Included in the Table are the weights of the pigs at the time of perfusion, the weight of the control (L) lung and the perfused (R) lung after perfusion and following squeezing. The volumes and histamine concentrations of the perfusion samples and the drainage fluid are also recorded. It is noted that in the case of the control animals 1:53 mill. to 1:3·5 mill. histamine was contained in the perfusion fluid in volumes from 47–62 ml. collected for 40 minutes. The drainage fluid measuring 0·7–4·2 ml. contained histamine varying in concentration from 1:6 mill. to 1:200,000. The output of histamine in the perfusion fluid varied from 0·9–15·6γ (av. 5·5γ) and in the drainage fluid from 0·1–8·5γ (av. 2·4γ). The combined output of both the perfusion and drainage fluid was 2·2–20γ, giving an average of 8γ histamine for the control experiments. There were ten perfusions in the control group. Of the "peptone" animals, the perfusion fluid following the injection of 10 mg. trypsin contained from 1:10 mill. to 1:1·25 mill. histamine in a volume of 34–57 ml. The drainage fluid contained from 1:1·9 mill. to 1:100,000 histamine in a volume of 0·8–2 ml. Thus the output of histamine in the perfusion fluid varied from 5·5–45·6γ (av. 21γ) and in the drainage fluid 1·1–11γ (av. 3·5γ) and the total output in the "peptone" cases was 6·6–46·7γ with an average output of 24·6γ for each lung perfusion. These findings are recorded in Table 2.

Now, it is noted that there is considerable variation in the output of histamine from experiment to experiment, and it has already been noted (Trethewie, 1939) that such figures are reduced more readily for comparison if the output is recorded in terms of the histamine content of the organ. This was the case following the injection of the same snake venom into different livers and estimating the histamine output. Therefore, it was considered advisable to relate the output obtained in these experiments to the histamine content of the lung: e.g., experiment six gives an output of 6·6γ from a lung containing 19γ histamine per gm. (Table 2) and experiment eight gives an output of 38·2γ from a lung containing 100γ histamine per gm. The ratio of output to histamine content is almost identical in this pair. Another variation is introduced by varying weight of the lungs and though this is minimized to a degree by the design of the experiment, the output should be related on these grounds to the available histamine in the lung which might be liberated, that is, the histamine output which is liberated to the histamine content in γ/gm. multiplied by the weight of the lung. Further, the output per unit of lung tissue will be related to the intensity of the trypsin injection, i.e. the amount of trypsin injected per gm. of lung. Thus we have the output recorded for comparison as:

$$\frac{\text{total output histamine } (\gamma) \times 1{,}000}{\text{histamine content } (\gamma/\text{gm.}) \text{ lung} \times \text{wt. lung} \times \dfrac{\text{mg. trypsin injected}}{\text{wt. lung}}}$$

$$\text{or } \frac{\text{total output}}{\text{histamine content } (\gamma/\text{gm.})} \times 100.$$

These figures are recorded in Table 3. It is noted that the variation in output

of the control group is 5–33 (av. 17·8), while the corresponding findings for the "peptone" group are 24–61 (av. 43·2), in spite of the fact that the histamine content of the "peptone" lungs is 10·9γ/gm. higher on the average.

TABLE 3.

*Output of histamine expressed as 1,000 × No. γ histamine released per total γ content of lung per mg. trypsin per gm. lung injected.*

| Experiment No. | Control outputs. | Experiment No. | "Peptone" outputs. |
|---|---|---|---|
| 2 | 25 | 6 | 35 |
| 5 | 17 | 8 | 38 |
| 7 | 7 | 11 | 61 |
| 9 | 33 | 12 | 24 |
| 10 | 20 | 24 | 58 |
| 13 | 22 | — | — |
| 17 | 9 | — | — |
| 20 | 30 | — | — |
| 21 | 5 | — | — |
| 22 | 10 | — | — |
| Av. | 17·8 | Av. | 43·2 |

Statistical analysis shows that this difference is highly significant. Thus, provided the control has been adequate, the injections of peptone have increased the ease with which trypsin releases histamine from the isolated perfused lung of the guinea-pig.

The average weight of the pigs in the control group was 679 gm. and of the pigs in the "peptone" group was 676 gm. The average weight of the left lung in the control animals was 1·72 gm. (variation, 1·39–2·25 gm.) and of both lungs 4·26 gm. (Variation, 3·09–6·01 gm.). The average weight of the left lung in the "peptone" animals was 1·94 gm. (variation, 1·54–2·40 gm.) and of both lungs 4·56 gm. (variation, 3·86–5·30 gm.). The slight increase in the weight of the "peptone" lungs may be due to remaining oedema fluid which was more difficult to remove following the repeated peptone injections. The average ratio of the weight of the left lung to the weight of the pig in the control group was 0·0255 (variation, 0·0196–0·0331) and in the "peptone" group 0·0289 (variation, 0·0237–0·0356).

### 3. *Histamine Content of the Lung.*

It was suggested in view of the increasing content of histamine in the lungs with age in untreated guinea-pigs (Trethewie, 1947a) and the increase in the extreme variation of two and a half times in this figure in animals aged 20 weeks compared with those at 8 weeks, that this might be due to external factors. In this experiment the influence of peptone on the histamine content of the lungs has been investigated. The findings are recorded in Table 4.

TABLE 4.

*Histamine content of lungs.*

| Pig. No. | γ/gm. left lung. | Total γ. Left lung. | Total γ. Whole lungs.* | γ/gm. animal |
|---|---|---|---|---|
| **Control.** | | | | |
| 2 | 17 | 38·3 | 102·2 | 0·150 |
| 5 | 38 | 74·2 | 210·9 | 0·327 |
| 7 | 39 | 68·3 | 165·8 | 0·226 |
| 9 | 50 | 87·0 | 203·0 | 0·333 |
| 10 | 54 | 102·6 | 237·6 | 0·365 |
| 13 | 91 | 161·1 | 407·7 | 0·595 |
| 17 | 65 | 90·4 | 236·6 | 0·338 |
| 20 | 24 | 34·6 | 86·6 | 0·129 |
| 21 | 42 | 59·6 | 123·6 | 0·194 |
| 22 | 41 | 62·3 | 144·3 | 0·186 |
| Av. | 46·1 | 77·9 | 191·8 | 0·285 |
| **"Peptone."** | | | | |
| 6 | 19 | 45·6 | 100·7 | 0·149 |
| 8 | 100 | 180·0 | 410·0 | 0·712 |
| 11 | 29 | 62·1 | 141·8 | 0·189 |
| 12 | 57 | 104·9 | 264·5 | 0·363 |
| 24 | 80 | 123·6 | 308·8 | 0·475 |
| Av. | 57 | 103·2 | 245·2 | 0·378 |

* Estimated as though the right lung (injected) initially contained the same histamine concentration as the left.

It is noted that the content of histamine in the control group varied from 17–91γ/gm. (av. 46·1γ) for the squeezed lung and in the "peptone" animals the value varied from 19–100γ/gm. with an average of 57γ. On the average there is a slight increase in the lung content (10·9γ/gm.). Statistical analysis shows ($t$, 0·78) that this finding is not significant and it is unlikely from these findings that if all the remaining animals were estimated for the histamine content of the lung, a significant difference would be found. Now, since there was such a considerable increase in the output of histamine (more than double) following perfusion and the injection of trypsin it was thought wiser, noting that 12 animals remained in each group, to keep these animals to determine if there was any variation in the ability of the animals to withstand toxic doses of histamine.

Included in Table 4 are the total content of histamine in the left lung, the total histamine content in the whole lungs and this last quantity also estimated as γ/gm. animal. Since Feldberg and Kellaway (1937) have shown that the content of the left and right lung is approximately the same, we are justified in estimating the content of histamine as the

weight of both lungs multiplied by the histamine concentration in the left lung. It is noted that the histamine content of the left lung varied from 34·6–161·1γ with an average of 77·9γ (control). "Peptone" animals gave a variation from 45·6–180γ (average 103·2γ). Thus there is an increase in the histamine content of 33 p.c. Statistically this is not significant ($t$, 1·2). Similarly the histamine content of the whole lungs varied from 86·6–407·7γ in the controls with an average of 191·8γ and in the "peptone" animals 100·7–410γ with an average of 245·2γ, an increase of 28 p.c. following peptone treatment. Statistically this increase is not significant ($t$, 0·94). When the histamine is estimated as γ/gm. animal, the variation in the "control" series becomes 0·129–0·595γ/gm. with an average of 0·285γ/gm. In the "peptone" series the variation was 0·149–0·712γ/gm. with an average of 0·378γ/gm., a rise of 33 p.c. Statistically these findings are not significant ($t$, 0·96). However, a much larger series might produce significant results.

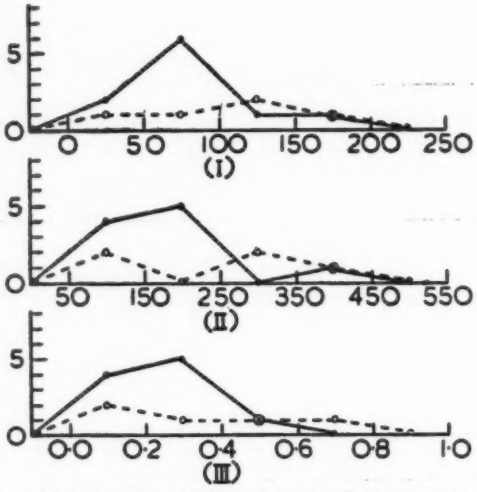


**Fig. 1.** Histamine content population curves. (i) Total content left lung (γ). (ii) Total content whole lungs (γ). (iii) γ/gm. animal.

Ordinate: Number of pigs in the range. Abscissa: Content histamine.

—— Control animals.
---- "Peptone" animals.

When we graph the histamine content of the lungs as total γ content left lung (Fig. 1 [i]), total γ content whole lungs (Fig. 1 [ii]) and total γ/gm. animal (Fig. 1 [iii]) it is noted that in every instance the "peptone" animals in spite of the fact that they are so few in number show a shift to the right. This is further indicated in Table 5, where in spite of the fact that there are only half as many "peptone" lungs as control lungs the upper half of the range contains more "peptone" animals than the control and the lower half of the range far more control figures. The ratio of control numbers $\frac{\text{lower half of range}}{\text{higher half of range}}$ varied from 4–9 to 1 while with the "peptone" numbers the ratio varied from 0·7–1·5 to 1.

TABLE 5.

*Number of animals in each group as obtained in Fig. 1.*

| | Range (γ). | Control. | "Peptone." |
|---|---|---|---|
| Total content left lung | 0–100 | 8 | 2 |
| | 100–200 | 2 | 3 |
| Total content whole lungs | 50–250 | 9 | 2 |
| | 250–450 | 1 | 3 |
| γ/gm. animal | 0·0–0·4 | 9 | 3 |
| | 0·4–0·8 | 1 | 2 |

When the total content of the whole lungs is examined as a two by two Table in this grouping statistical analysis shows that this difference is significant ($p$, 0·03). This means there is a significantly larger number of animals with high histamine contents of the lungs following peptone injections than is the case with the controls.

4. *Toxicity of Histamine Following Treatment.*

Since it has been shown that histamine is liberated by peptone (Feldberg and O'Connor, 1937), it is not inconceivable that following repeated injections of peptone by releasing histamine we might increase the tolerance of the animal to histamine. This is also important, since histamine is more readily liberated by trypsin following peptone injections (this paper) and, unless there is a change in tolerance for histamine, it would seem that such "shock" treatment would be harmful. The remaining 24 animals (12 controls and 12 in the "peptone" group) therefore were used to estimate any change in the toxicity of histamine following peptone treatment. At first four control pigs were injected into the jugular vein with 0·39 mg. histamine/kg. and all survived. However, it was thought inadvisable to determine the lethal dose of histamine following injection into the jugular vein, in spite of the fact that this gives even absorption, since struggling on the part of the animal is almost certainly followed by an output of adrenalin which is a histamine antagonist.

TABLE 6.

*Toxicity of histamine injected subcutaneously following treatment.*

| "Peptone" animals. | | | Control animals. | | |
|---|---|---|---|---|---|
| Dose histamine mg./kg. | Numerator: Deaths. Denominator: Total No. | Death times (min.). | Dose histamine mg./kg. | Numerator: Deaths. Denominator: Total No. | Death times (min.). |
| | | | *4·1 | 0/4 | — |
| | | | *6·6 | 2/4 | 46, 71+ |
| 8·2 | 1/4 | 26 | 7·5 | 2/4 | 69, 71 |
| 9·6 | 3/4 | 28, 27, 40 | 9·6 | 3/4 | 33, 20, 17 |
| †11·0 | 3/3 | 37, 27, 17 | *11·0 | 4/4 | 25, 21, 29, 29 |

† The fourth animal in this group died of asphyxia just prior to injection.

* Animals from the same source, and of the same weight and age as the controls, untreated.

By the method of probits an estimate of the l.d.$_{50}$ was made using groups of four animals for each dose. For the reason stated above, injections of histamine were made subcutaneously, but care had to be exercised to select an area of the abdomen which was not affected by the the previous peptone injections. This created difficulty but it was thought that this was preferable to jugular injection. The animals were observed for a period of 48 hours.

In the control groups of animals a dose of 9·6 mg. histamine/kg. killed three out of four guinea-pigs, and 7·5 mg./kg. killed two out of four. In addition, it was thought

justifiable to include twelve more animals obtained from the same source and of the same weight and age as the controls to enlarge the series to obtain thereby a better estimate of the l.d.$_{50}$ of the control animals. A dose of 11 mg. histamine/kg. killed four out of four, and 6·6 mg. histamine/kg. killed two out of four while 4·1 mg./kg. was non-lethal. These findings are recorded in Table 6.

The first group of "peptone" animals received 8·2 mg. histamine/kg. since it was anticipated that there would be an increase in the l.d.$_{50}$ of histamine for these animals. Only one out of four animals died after receiving this dose. However, when 11 mg. histamine/kg. were given there were three out of three deaths. The first animal of this group had died of asphyxia immediately prior to the injection of histamine thus reducing the group to three in number. Therefore, the next dose given was 9·6 mg. histamine/kg. and three out of four of the animals died. These findings are also recorded in Table 6.

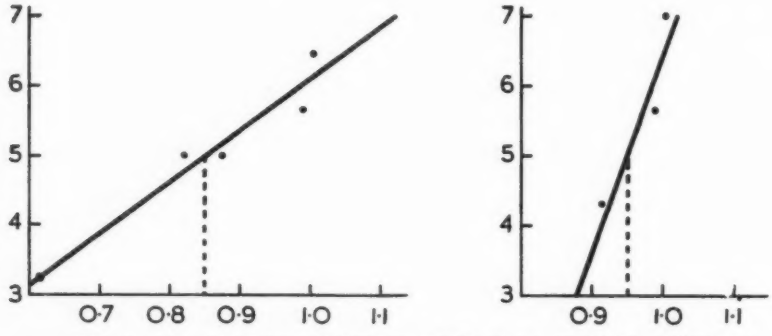


**Fig. 2.** l.d.$_{50}$ histamine. Ordinates: Probit of p.c. mortality. Abscissae: Log. dose histamine (mg./kg.). Left hand figure: Control. Right hand figure: "Peptone."

When these deaths are recorded by the method of probits (Fig. 2) an estimate of the l.d.$_{50}$ is obtained indicating that the lethal dose of histamine by subcutaneous injection is 7·1 mg./kg. for controls and 8·9 mg./kg. for the "peptone" pigs. The probit points were estimated by the method of Bliss (1935).

It is surprising that there has been no considerable change in the tolerance of the animals to histamine injections following repeated treatments with peptone. It is possible that the injury to the abdominal skin by the injections of peptone clouded the result. However too much significance must not be attached to these findings, though we have probably gained as much information as possible from the small groups investigated.

## DISCUSSION.

It has been shown that peptone causes the release of histamine from the isolated lung of the guinea-pig following intra-arterial injection (Feldberg and O'Connor, 1937), and for this reason repeated injections of peptone have been made into guinea-pigs used in this work. Seven injections in all were made,

which act in these animals as an injurious agent liberating histamine. Control animals were injected with Tyrode only. It was desired to determine if previous experience had any influence on the content of histamine in the lung and on the ease of liberation of histamine. In this way it was hoped to obtain information of immunological importance.

First we will consider the output of histamine from the perfused lungs of these animals following the injection of trypsin. Rocha e Silva (1940) has already shown that trypsin causes the liberation of histamine. A dose of 10 mg. was injected into the perfused right lung so that with this small dose any difference in the ease of liberation might more readily be detected, and for the same reason perfusion was only continued for 40 minutes. Though histamine is liberated into the tissue spaces mainly (Trethewie, 1945), and in fact this forms the basis for the thesis regarding tissue injury elaborated by Feldberg and Kellaway (1937), the lung was hyperventilated to obtain the perfusate so that as much as possible of the liberated histamine could be detected. It is well recognized, however, that this is largely tissue-space histamine and would not form intravascular histamine to any extent in the intact animal. For the same reason, the drainage fluid was collected in each instance for, while in simple control perfusions with Tyrode, the output of histamine is negligible in the drainage fluid in an experiment of similar duration without injection of an injurious agent, the drainage fluid obtained following tissue injury contains quite considerable concentrations of histamine.

It was noted that the output of histamine in the perfused fluid from the "peptone" animals averaged 21γ and the drainage fluid averaged 3·5γ. The average of the total "peptone" output was 24·6γ. In the case of the control perfusions, however, the output in the perfusion fluid only averaged 5·5γ, and in the drainage fluid 2·4γ with an average total output of 8γ. Thus there is a considerably greater output of histamine, especially in the perfusion fluid with the "peptone" animals. However, since the average histamine content of the organs was greater in the case of the "peptone" animals, this difference is possibly greater than might occur were the histamine content the same. For example, it has been shown (Trethewie, 1939) that the output of histamine following the injection of black snake venom is almost proportional to the histamine content of the organ within the limits of experimental variation. Since we have not evidence of a statistically significantly greater histamine content in the "peptone" animals, this correction should be made in case such a fortuitous occurrence might indicate an increased output of histamine in the "peptone" animals when it is only due to a natural variation in the histamine content of the organs studied. When this is done and the output is compared in relation to the histamine content of the organ, further details of which have been amplified in Section 2, the average output of histamine in the case of the control animals becomes 17·8 units and in the "peptone" animals 43·2 units.

This difference is highly significant statistically. Thus, even when a correction is made for the histamine content of the organ, the output of histamine is greater in the case of the "peptone" animals. This can only be held due to different treatment of the "peptone" animals. So far as we know, the only difference has been that they have been injected with peptone instead of Tyrode, though experimental variation may explain it in part. A bias may have been created by the greater number of deaths before perfusion in the "peptone" group (8) compared with the control group (3), though this difference is not significant ($p$, 0·09).

It then becomes evident that previous repeated injections have increased the ease with which histamine is liberated by another injurious agent. This is probably an expression of the principle of training, increasing the ability of a cell to perform a specific function.

It has been noted (Orr Reynolds, 1947) that such a phenomenon as causalgia may be related to previous experience. For example, it has been shown that if teeth are filled without an anaesthetic, post-filling pain sensitivity phenomena frequently occur. Such is not the case where an anaesthetic has been used. It is as though the nerve tissue has been trained to lower its threshold to stimuli. It is not inconceivable that a similar principle may be involved in the rationale of the treament of insanity. Shock therapy may act by enabling the patient to forget the last straw which broke his mental back (Edwards, 1947). Evidently the nerve tissue has been educated to respond to unfavourable stimuli more readily than the normal as in extreme mental activity in mania, and if the organism can be made to forget these later episodes a degree of normality may be restored. Adey suggested (1944) that the value of shock treatment lay in the sleep which followed such treatment and not in the convulsion. An apparatus was made and electro-narcosis tests were carried out on animals (Rose and Rabinov, 1945). It seemed that a weapon was available to test the validity of the suggestion that improvement in shock treatment of mental cases was due to the sleep and not to the convulsions induced since this apparatus produced sleep without convulsion. Having carried out successful electro-narcosis on monkeys it was thought advisable to test electro-narcosis on patients suffering from mania since convulsion therapy was being used in these cases. This was performed by one of us (E.R.T.). Attempts were unsuccessful as regards improvement but were not followed up. Also at this time a paper then appeared (Frostig *et al.*, 1944) which produced evidence indicating the value of electro-narcosis in schizophrenia. This supports Adey's contention. Further it indicates a principle which may involve many different activities of a cell, cells being known to perform very many functions though certain cells have particular ones more highly developed. Impulse conduction and histamine release may have this in common. Training may make further responses more ready and when stimuli which would take these pathways are stopped for a time, the "learning" may be "unlearned." A similar activity may take place in anaphylaxis. Other phenomena are related. In a loop of intestine deprived of pancreatic juice the secretion of enterokinase gradually fails (Sawitsch, 1904). Repeated stimulation of the vagus nerve causes an increase in the response to a stimulus of the muscle of the intestine (Bayliss and Starling, 1899) even though this is not associated with an increase in the output of acetylcholine (Trethewie, 1942). Thus facilitation evidently occurs also in these instances.

Having produced evidence that there is an increased output of histamine following the injection of a known injurious agent after recovery from a previous injury, the effect of this on the animal requires consideration. At first it might

be thought that this is a harmful effect, owing to the fact that a highly toxic substance such as histamine is more readily liberated. However, it has just recently been shown (Miklós Jancsó, 1947) that histamine activates the macrophages. In fact, it is suggested that this is the "function" of histamine as distinct from its inimical effects. The results are consistent with the hypothesis that ease of liberation of histamine in response to an injurious stimulus is developed as a result of past experience of response to fortuitous or experimental stimuli of the same general type, e.g. minor bacterial infections or peptone injections. The experimental findings fit with those of Jancsó into a very interesting pattern. They indicate that histamine liberation, as has been suggested by Burnet (1940), may play an important part in the reactions to local trauma and infection, by increasing local blood supply and capillary transudation and activating circulating and tissue macrophages to phagocytic activity. It would be quite in keeping with general physiological principles that, if histamine liberation is a useful function in such common emergencies, it will become more readily initiated with "training." In a similar way another "emergency mechanism," antibody production, responds much more effectively to a later stimulus than to the first. In all probability other aspects of the "emergency response" to local infection would also behave similarly. If, now, by liberating more histamine the reticulo-endothelial system cells are increased in activity, it may be that this favourable action more than outweighs the harmful effect. If the toxicity were reduced and the macrophage activity unchanged, the increased histamine released might be a valuable effect. We have only slight evidence of decreased toxicity of histamine in the "peptone" animals. If the slope of the estimates obtained means anything, it suggests that there is a considerable difference in the toxicity of smaller doses of histamine.[2] However, too many conclusions must not be drawn from such a small series. There is some evidence that the harmful effect, at least in less severe cases, may not be nearly so apparent. It has already been shown (Trethewie, 1945) that histamine is liberated into the tissue spaces and does not usually find its way into the vessels to a large extent in pneumonia in the cat. Provided the pulmonary effects are not so gross as to produce systematic effects, this is not inimical. It may be relevant to this to note that Gerard, Tobias *et al.*, found that goats which had survived a fairly severe phosgene exposure (somewhere around the $l.d._{50}$) were subsequently able to withstand progressively heavier dosages until a tenfold stronger dose led to no further mortality. The mechanism of this was not fully worked out, but some evidence was produced that simple selection of the more resistant animals was one factor. Recently Box and Cullumbine (1947) suggested that the greater resistance to a second exposure was due to a diminished depth of breathing. This may also be a factor, though Gerard (1947) doubts if it is the whole story.

[2] Karády and Kovács have just reported in *Nature* (1948, 161, p. 688) evidence that increased resistance following injury is due to formation of a substance they call "resistine."

The importance of the tissue spaces has already been elaborated in investigations on the liberation of histamine by phosgene (Trethewie, 1947b, c). It was shown that there was a parallelism between the lethal doses of inhaled phosgene and histamine when given by venous injection. Gerard (1947) pointed out that if it is considered that the harmful effect of histamine in phosgene poisoning is produced by the local effect in the lung producing oedema, one should not necessarily anticipate a parallelism between the lethal dose (i.v.) of histamine and inhaled phosgene. However, it is undoubtedly the case that oedema of the lung is the common cause of death primarily with phosgene. Now if the lethal effect of histamine is only an index as it may well be, of its ability to cause local injury then it is not necessarily so surprising that there is a parallelism between the toxic effect of intravascular histamine and inhaled phosgene. Further, there is another variable operative here in addition to species differences in the toxic effect of histamine, namely, the varying ability of phosgene to release histamine in the different types of animals. Cordier (1947) has shown that guinea-pigs submitted to pre-treatment with histamine have a lower mortality than untreated when exposed to phosgene. This is in favour of the contention that histamine plays a part in phosgene poisoning though Cordier points out that such treatment increases the tolerance to other procedures. Since the mechanism of damage in these other procedures is not known, such evidence is incomplete. Further, Cordier states that there is a short and early release of histamine in phosgene poisoning. This is not the case with a low c.t.[3] (2,100) (Trethewie, 1947b) where with the guinea-pig, histamine does not appear for four to five hours. It is this delayed rise which is important.

In an earlier paper (Trethewie, 1947a) it was found that after an initial fall following birth, the histamine content of the lung, when expressed in $\gamma$/gm. organ or total $\gamma$ content or $\gamma$/kg. animal, showed a slight rise with age and more especially showed an increase in the extreme variation of the histamine content, e.g. at 20 weeks the extreme variation increased two and a half times from that at 8 weeks. It was suggested that an attempt should be made to determine if the histamine content was influenced by outside factors. With this in mind, the histamine content of the lung was determined in these investigations. There was an increase in the mean content (10·9 $\gamma$/gm.) of the lung in the "peptone" animals compared with the controls. This was not significant and since $t$ tests indicated significance was remote, it was thought more advisable to investigate the remaining animals for toxicity of histamine, since this might be important in relation to the release of histamine. Further, the average histamine content ($\gamma$/gm. lung) of the control animals, when each family was compared, was slightly higher than the "peptone" animals. However, there were only three family comparisons possible.

When the total histamine content of the lungs was compared (Table 5) it was found that there was a significantly greater proportion of the animals in the upper half of this range than the lower in the group receiving peptone. Evidently some animals in the absence of peptone injections will develop an equally high histamine content in the whole lungs. When peptone is given, more animals achieve this level. It is possible, therefore, that there is an unknown factor responsible for the rise of histamine content in the lung in the natural

[3] c.t. = concentration (mg. per cubic metre) × time of exposure (mins.)

course of events, which we will call factor A. A known factor (factor B), such as peptone injections, may also cause the histamine content to rise, giving a shift to the population curve. Since there is not in this small series a significantly higher histamine content in all the lungs taken as one group, it is possible that factor A and factor B act through an intermediary which is the mechanism responsible for increased histamine formation. In this way when saturation, so to speak, is produced, little more histamine can be formed even in the presence of continued stimulation (Fig. 3). The diagram indicates a control series (upper) of cells subject to influence A with one cell responding by histamine production (cross-hatch) and a lower ("peptone") group showing one cell responding to factor B and one responding to the combined effect of A and B.

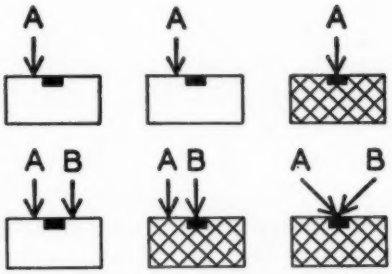


**Fig. 3.** Schematic illustration of the influence of factors A and B on histamine formation in the cell.

Upper cells: Control cells subject to influence of factor A with one cell responding by histamine production (crosshatch).

Lower cells: "Peptone" group showing one cell responding to factor B and one responding to the combined effect of A and B.

Another point of interest is whether these findings are relevant to the phenomenon of over-reaction to general infectious disease in humans of the 15–35 age group. This susceptibility of the young adult, particularly to infections which are not widely endemic has been particularly discussed in relation to the influenza pandemic of 1918–19. Epidemiologically, the young adult incidence of mortality was the most important distinguishing feature of the pandemic. It has been suggested (Burnet and Clark, 1942) that in general "the various mechanisms by which inflammatory responses are called into play become on the whole more reactive and more efficient up to the prime of life" but that this increased reactiveness is only of survival value in regard to local, especially traumatic, infections. It is a positive danger to infections generalized through the body or involving an extensive organ like the lung. It has still to be determined whether this changed reactivity of human beings is associated with increased ease of liberation of histamine and associated pharmacologically active substances, and if this is the case, whether the increasing ease is a result of recurrent fortuitous training in histamine liberation from the inevitable casual infections. The steady loss of reactivity in the present sense in individuals from the age of 35 to 40 onwards would have to be interpreted in the light of what conclusions were reached about the changes associated with adolescence and young adult life, the alternatives being a diminished reactivity to histamine-

liberating stimuli or a diminished reactivity of the tissues to liberated histamine with the further alternatives that the altered condition was a simple, genetically-conditioned result of ageing or depended on some type of desensitizing action requiring repeated histamine liberation by appropriate stimuli.

## SUMMARY.

The output of histamine from the isolated lung of the guinea-pig following the injection of trypsin was significantly greater in those animals which had previously received repeated subcutaneous injections of peptone.

The total content of histamine in the lungs was somewhat (but not significantly) higher in "peptone" than in control animals, and when the total content of histamine in the whole lung was estimated, there was a significantly greater number of "peptone" animals in the upper half of the range than of control animals.

The significance of these findings, especially from the immunological standpoint, is discussed.

## REFERENCES.

Adey, J. K. (1945): private communication.
Bayliss, W. M. and Starling, E. H. (1899): J. Physiol., 24, p. 99.
Bliss, C. I. (1935): Ann. applied Biol., 22, p. 134.
Box, G. E. P. and Cullumbine, H. (1947): Brit. J. Pharmacol., 2, p. 38.
Burnet, F. M. (1940): Biological Aspects of Infectious Diseases, Monographs of Walter and Eliza Hall Institute.
Burnet, F. M. (1942): Influenza, Monographs of Walter and Eliza Hall Institute.
Cordier, d'A. P. (1947): Private communication. Ministry of Supply, London.
Edwards, K. F. (1947): Med. J. Austral., 2, p. 512.
Feldberg, W. and Kellaway, C. H. (1937): J. Physiol., 90, p. 257.
Feldberg, W. and O'Connor, W. J. (1937): *Ibid.*, 90, p. 288.
Frostig, J. P., van Harreveld, A., Rezniek, S., Tyler, D. B. and Wiersma, C. A. G. (1944): Arch. Neurol. Psychiat., 51, p. 232.
Gerard, R. W. (1947): Private communication.
Gerard, R. W., Tobias, J. M., *et al.* (1947): Private communication.
Jancsó, Miklós (1947): Nature, 160, p. 227.
Reynolds, Orr (1947): Quoted by Gerard (1947).
Rocha e Silva, M. (1940): Arch. exp. Path. u. Pharmak., 194, p. 335.
Rose, S. and Rabinov, D. (1945): Med. J. Austral., 1, p. 657.
Sawitsch, W. W. (1904): Quoted by Babkin (1928): Die Äussere Sekretion der Verdauungsdrüsen, 2te Auflage. J. Springer, Berlin.
Trethewie, E. R. (1939): Austral. J. exp. Biol., 17, p. 145.
Trethewie, E. R. (1942): *Ibid.*, 20, p. 9.
Trethewie, E. R. (1945): *Ibid.*, 23, p. 49.
Trethewie, E. R. (1947a): J. Immunol., 56, No. 3, p. 211.
Trethewie, E. R. (1947b): Med. J. Austral., 1. p. 612.
Trethewie, E. R. (1947c): *Ibid.*, 1, p. 746.

# THE MECHANISM OF THE HYPOGLYCEMIC ACTION OF ALLOXAN

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Hughes, Ware and Young (1944) have suggested that the profound hypoglycemia following initial hyperglycemia after injection of alloxan is due to a slow release of insulin from dead or dying islet cells. In support of this, Ridout, Ham and Wrenshall (1944) have found that the insulin content of the pancreas does not fall appreciably until after most of the islet cells are dead, as indicated by histological examination. Banerjee (1945) has demonstrated that hypoglycemic convulsions do not occur after intravenous injections of alloxan in partially depancreatized rabbits, whereas hypoglycemia and convulsions occur in rabbits with intact pancreas. Goldner and Gomori (1944), Ridout *et al.* (1944), Houssay, Orías and Sara (1945) found that alloxan exerts no hypoglycemic action in animals possessing little or no islet tissue due either to previous pancreatectomy or to previous injection of alloxan. All this evidence indicates that alloxan hypoglycemia is pancreatic in origin and probably is due to release of pre-formed insulin from islet cells damaged by alloxan. Houssay *et al.* (1945), however, found that profound hypoglycemia occurs in chloralosed dogs, from which the pancreas had been removed half an hour previously, one to seven hours after intravenous injection of alloxan. Consequently they denied that the pancreas is involved in the occurrence of alloxan hypoglycemia. In view of this conflicting evidence the problem of the mechanism of alloxan hypoglycemia has been re-investigated, using the guinea-pig as an experimental animal for reasons given below.

## METHODS.

*Animals.*

Experiments on alloxan hypoglycemia were carried out on adult male guinea-pigs and on wild rabbits obtained as young animals of 200–300 gm. weight and raised in the laboratory. These guinea-pigs and rabbits were fed a standard diet and fresh cabbage daily except in one series of experiments where guinea-pigs were kept on the methionine and cystine deficient diet described by Haag and Wright (1940). 0·2 p.c. methionine was added to this diet for

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control animals. 0·1 p.c. ascorbic acid was added to both deficient and control diets. All animals were fasted 17 hours before experiments.

*Alloxan Injections.*

Dosages of 200 mg./kg. body weight were used throughout. Injections were made either subcutaneously, intraperitoneally or intravenously, in rabbits. It was not possible to inject unanaesthetized guinea-pigs intravenously so alloxan was injected intracardially, intraperitoneally or subcutaneously. In one series of experiments on guinea-pigs alloxan was injected, after preliminary laparotomy under "Avertin" anaesthesia, directly into the pancreas or liver. The incision was then closed with interrupted sutures. Control injections of water were made in a similar manner.

*Pancreatectomy.*

This operation was carried out on rabbits under "Avertin" anaesthesia. Owing to the diffuse nature of the organ, removal was not complete, but physiological removal of the remnants was achieved by tying off the arteries and veins supplying the pancreas. The incision was closed with interrupted sutures. Control operations consisted of laparotomy and manipulation of the pancreas. The animals in both cases quickly regained consciousness and alloxan was injected intravenously half an hour after removal of the pancreas.

*Extraction and Assay of Pancreatic Insulin.*

Total pancreatic tissue was removed, extracted and crude insulin prepared according to the method of Jephcott (1932). This insulin was taken up in N/300 HCl and the potency determined by a cross-over test on four rabbits carried out by the method of Marks and Pak (1936).

*Chemical.*

Blood sugar determinations were made on venous blood by the Hagedorn-Jensen method. Blood reduced-glutathione was determined by the method of Woodward and Fry (1932).

*Histological.*

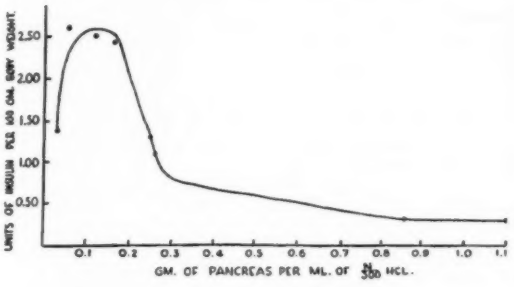
Pancreas and liver tissues from guinea-pigs were fixed and stained by the method recommended by Richardson (1940). Impregnation, however, was carried out with Fisher "Tissuemat."

## RESULTS.

*Insulin Content of the Pancreas of the Guinea-Pig.*

Before giving the main results of this investigation, it will be necessary to describe some experiments carried out to determine the amount of pancreatic insulin in guinea-pigs. Marks and Young (1940) using the mouse convulsion method of assay, reported that the insulin content of guinea-pig pancreas was found to be 0·07 units/100 gm. body weight, whereas the figures for rabbits were from 1·17 to 1·42 units/100 gm. They showed that a substance or substances in the extract of guinea-pig pancreas interfered with absorption of insulin from subcutaneous tissues but thought it improbable that such an effect could entirely account for the low figure found. However, it has been found by the writer that when the crude insulin from guinea-pigs' pancreas was taken up in amounts of N/300 HCl equivalent to dilutions of 0·05 to 0·16 gm. of fresh pancreas per ml. the values found were 2·50, 2·51, and 2·40 units/100 gm. (See Fig. 1). If, however, the solutions were made stronger the potency was found to decrease until with a dilution of 1·10 gm. fresh pancreas/ml. a value of 0·30

units/100 gm. was obtained. The average percentage reductions with the high dilutions and low dilutions were approximately the same (28·0). Dilution in excess of 0·05 gm. fresh pancreas/ml. gave markedly decreased percentage reductions.



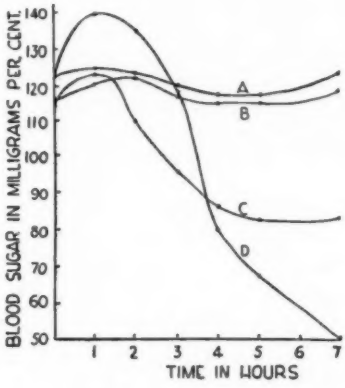




**Fig. 1.** The relationship between amount of insulin detectable and the degree of dilution of guinea-pig crude-insulin preparations.

**Fig. 2.** Curve A. Average response of 12 guinea-pigs to subcutaneous injection of 200 mg./kg. alloxan.

Curve B. Average response of 4 guinea-pigs to intracardial injection of 200 mg./kg. alloxan.

Curve C. Average response of 6 rabbits to subcutaneous injection of 200 mg./kg. alloxan.

Curve D. Response of 6 rabbits to intravenous injection of 200 mg./kg. alloxan.

It is apparent from the above that a substance in guinea-pig crude-insulin, when in sufficient concentration, interferes with insulin absorption from subcutaneous tissues, and when diluted no longer inhibits absorption thus giving a truer estimation of the potency.

Assays of pancreatic insulin from two batches of three rabbits whose average weight was the same as that of the guinea-pigs, gave values with high dilutions of 1·32 and 1·88 units/100 gm. body weight. Thus the guinea-pig pancreas contains as much or more insulin than that of the rabbit.

*Comparison of the Effect of Alloxan on Blood Sugar Levels of the Guinea-Pig Compared with the Effect on those of the Rabbit.*

Subcutaneous or intracardial injection of 200 mg./kg. of alloxan has practically no effect on the guinea-pig's blood sugar levels (Fig. 2, curves A and B). Subcutaneous or intravenous injection of the same dose of alloxan in rabbits brings about an initial hyperglycemia followed by hypoglycemia and sometimes convulsions (Fig. 2, curves C and D).

Lazarow's (1946) observation that injection of reduced glutathione inhibits the action of alloxan on blood sugar levels, offers a possible explanation for the absence of any effects in guinea-pigs.

A series of blood reduced-glutathione determinations was made on rabbits and guinea-pigs. The results are given in Table 1. The average value for the guinea-pig's reduced-glutathione (experiment 3) was found to be considerably greater than that for rabbits (experiment 1). By the *t*-test the difference between these average figures was highly significant ($P > 0{\cdot}001$).

TABLE 1.

*Blood reduced-glutathione of rabbits and guinea-pigs under various conditions.*

| Experiment No. | Animal. | Number. | Treatment. | Blood reduced-glutathione in mg./100 ml. |
|---|---|---|---|---|
| 1 | rabbit | 12 | Given standard laboratory diet + fresh cabbage | 38 |
| 2 | rabbit | 4 | Given standard laboratory diet + fresh cabbage and injected subcutaneously with 200 mg./kg. reduced glutathione. Blood taken 15 mins. after injection. | 65 |
| 3 | guinea-pig | 12 | Given standard laboratory diet + fresh cabbage | 58 |
| 4 | guinea-pig | 3 | Given methionine and cystine deficient diet for 3–4 weeks. | 40 |
| 5 | guinea-pig | 3 | Given methionine and cystine deficient diet + 0·2 p.c. methionine for 3–4 weeks | 56 |

It is possible that this relatively high blood reduced-glutathione inhibits the action of alloxan in guinea-pigs.

The following experiments substantiate the possibility. Fifteen minutes after subcutaneous injection of 200 mg./kg. of reduced glutathione the level of rabbits' blood reduced-glutathione was comparable to that of guinea-pigs (Table 1). Intraperitoneal injection of alloxan now had no effect on the rabbits' blood sugar levels (Fig. 3, curves A and B). When guinea-pigs' blood reduced-glutathione was lowered, by feeding a methionine and cystine deficient diet, to a level comparable with that of rabbits the guinea-pigs showed the hypoglycemic effect of alloxan (Fig. 3, curve C). Addition of methionine to the diet kept up the level of glutathione, and the blood sugar levels of the guinea-pigs on this diet showed little change after alloxan (Fig. 3, curve D).

*Effect of Alloxan Injected directly into the Pancreas or Liver of the Guinea-pig.*

From the data in Fig. 4 it is apparent that injection of alloxan throughout the liver of the guinea-pig left the blood sugar levels virtually unchanged. On the other hand injection of alloxan into one portion, or into several regions of the pancreas, resulted in hypoglycemia, which was marked in the latter case and sometimes convulsions occurred. Control injections of water into the pancreas left blood sugar levels unchanged (Fig. 4, curve D).

Five days after injection the pancreas was taken from the surviving animals (those injected in the pancreas in several places with alloxan failed to survive more than 24 hours). Macroscopical examination of pancreas injected in one place showed half the pancreas to be a greenish-grey colour; the uninjected portion looked the normal pinkish colour. Cytological examination revealed the grey portion to be necrosed, both acinar and islet tissue being involved. The islet tissue consisted of non-cellular debris surrounded by necrosed acinar cells (Fig. 5a). No insulin could be detected in this portion of the pancreas (Table 2). The other half of the pancreas showed no damage in the acinar tissue, and except for degranulation of beta cells in some islets, relatively intact islet tissue (Fig. 5b). A considerable amount of insulin was found in this portion (Table 2). Islet tissue in the guinea-pigs injected in the liver with alloxan, and in the pancreas with water, was normal (Fig. 5c and d) and the insulin content in both cases was relatively unchanged.

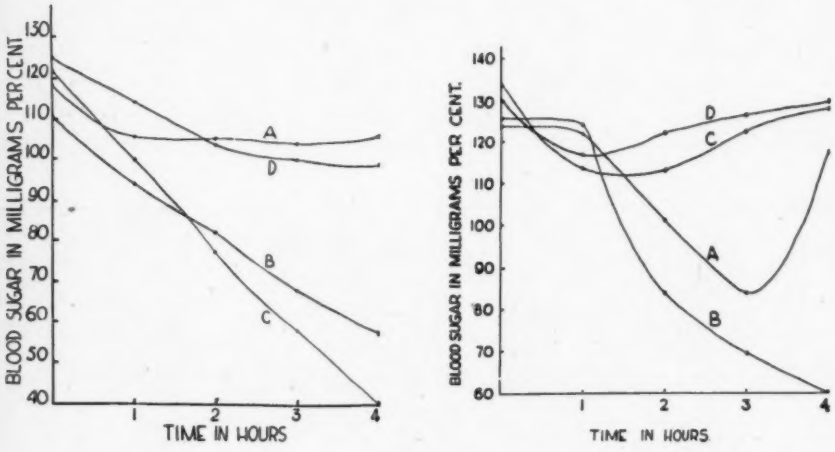



**Fig. 3.** Curve A. Average response of 3 rabbits which had been injected subcutaneously with 200 mg./kg. glutathione 15 minutes previously, to intraperitoneal injection of 200 mg./kg. alloxan.

Curve B. Controls to above experiment. Response of 4 rabbits to intraperitoneal injection of 200 mg./kg. alloxan.

Curve C. Average response of 3 methionine and cystine deficient guinea-pigs to intraperitoneal injection of 200 mg./kg. alloxan.

Curve D. Average response of 3 guinea-pigs given supplement of 0·2 p.c. methionine in methionine and cystine deficient diet, to intraperitoneal injection of 200 mg./kg. alloxan.

**Fig. 4.** Curve A. Average response of 3 guinea-pigs to an injection of 200 mg./kg. alloxan in 1 ml. of water, into one portion of the pancreas.

Curve B. Average response of 6 guinea-pigs to injections of alloxan (total of 200 mg./kg. in 1 ml. water) throughout the pancreas.

Curve C. Average response of 6 guinea-pigs to injections of alloxan (total of 200 mg./kg. in 1 ml. water into the liver.

Curve D. Average response of 3 guinea-pigs to injections of water (total of 1 ml.) throughout the pancreas.

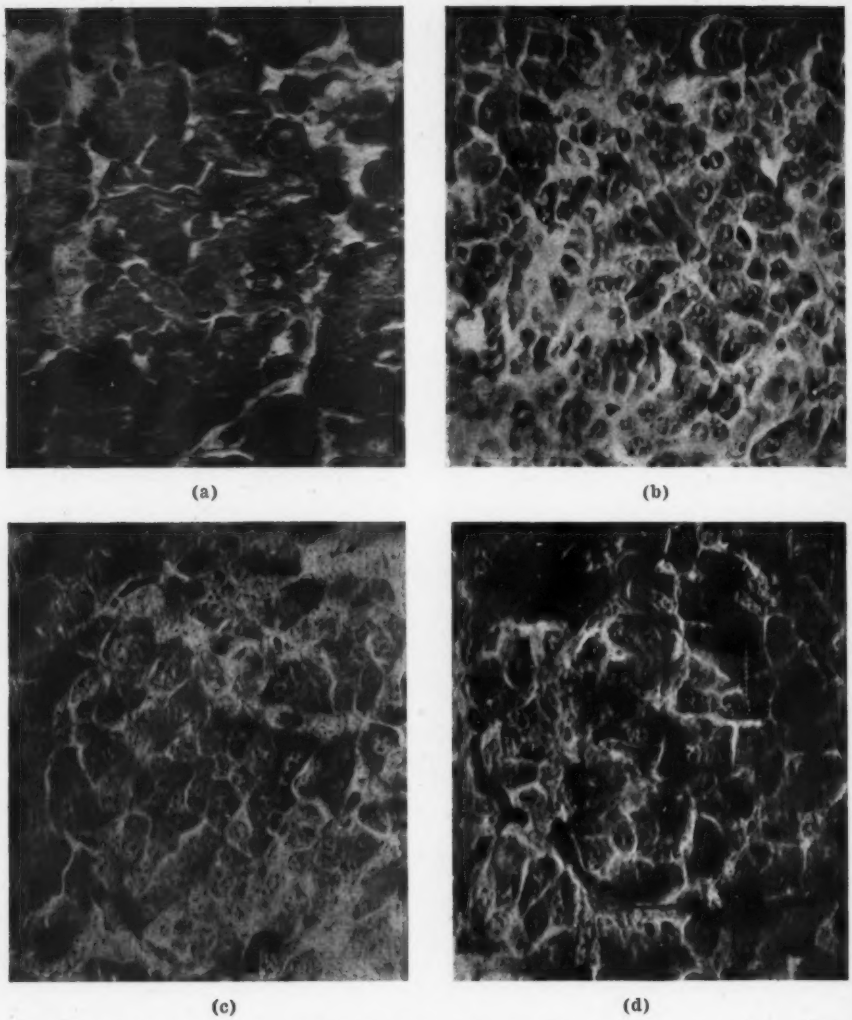


**Fig. 5.** (a). Islet of Langerhans in that portion of guinea-pig pancreas injected with alloxan. Nuclei have disappeared and the surrounding acinar tissue is necrosed.

(b). Islet in same pancreas in the portion not injected with alloxan. Some beta cells are degranulated. Acinar tissue is normal.

(c). Normal islet in pancreas of guinea-pig injected in the liver with alloxan.

(d). Normal islet in guinea-pig injected in the pancreas with water.

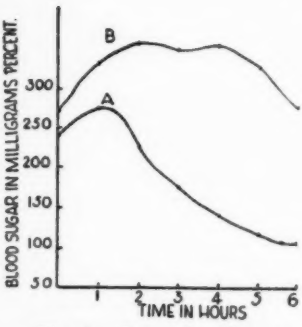



**Fig. 6.** Curve A. Average response of 3 sham-pancreatectomized rabbits to intravenous injection of 200 mg./kg. alloxan.

Curve B. Average response of 3 pancreatectomized rabbits to intravenous injection of 200 mg./kg. alloxan.

*Effect of Alloxan on Blood Sugar Levels of the Rabbit Depancreatized Half-an-Hour Previously.*

Under the conditions described, the blood sugar of control and depancreatized rabbits was elevated considerably. The response of the controls to intravenously injected alloxan was typical, however. An initial rise in blood sugar above control level occurred followed by a marked fall (Fig. 6, curve A). The depancreatized rabbits showed only hyperglycemia (Fig. 6, curve B).

## DISCUSSION.

Under the conditions described it is apparent that alloxan when injected intracardially or subcutaneously into normal guinea-pigs has no hypoglycemic action. The observation that alloxan injected into the pancreas of this species causes hypoglycemia and does not when injected into the liver, strongly suggests that the hypoglycemia is pancreatic in origin and not hepatic as stated by Houssay, Orías and Sara (1945). The fact that guinea-pig normal pancreas has an ample store of insulin and that the portion injected with alloxan has none is in accordance with other evidence that alloxan hypoglycemia is due to liberation of pre-formed insulin by disintegrating islet cells.

TABLE 2.

*Insulin content of guinea-pig pancreas after injection of alloxan or water into pancreas or liver.*

| Treatment. | No. of pancreas. | Insulin content in units/gm. of pancreas. | |
|---|---|---|---|
| | | Injected portion. | Uninjected portion. |
| 200 mg./kg. of alloxan in 1 ml. $H_2O$ injected into one portion of pancreas | 2 | Nil<br>Nil | 2·72<br>3·80 |
| 200 mg./kg. of alloxan in 1 ml. $H_2O$ injected into liver | 2 | 3·75<br>2·31 | |
| 1 ml. of water injected into pancreas | 1 | 3·83 | |

Mention has already been made of Lazarow's work on glutathione inhibition of alloxan and its bearing on the absence of hypoglycemia in guinea-pigs. Leech and Bailey (1945) and Brückmann and Wertheimer (1947) have shown that alloxan itself markedly lowers the amount of reduced glutathione in the blood of mammals. Apparently this property is closely associated with the capacity of alloxan to destroy islet tissue, since uric acid (structurally related to alloxan), although non-diabetogenic in normal animals, is diabetogenic when blood reduced-glutathione is lowered by feeding of methionine and cystine deficient

diet (Griffiths, 1948). It would seem then that the initially higher blood glutathione of the guinea-pig prevents alloxan lowering glutathione to a level sufficient to allow it to exert its necrotic effect on the islets.

The finding that alloxan does not induce hypoglycemia in recently pancreatectomized rabbits is not in agreement with the work of Houssay *et al.* (1945). Whilst the writer's work was in progress Goldner and Gomori (1947) reported that they also could not repeat Houssay's work. Goldner and Gomori also confirmed Banerjee's (1945) finding that alloxan does not induce as severe a hypoglycemia in partially depancreatized rabbits as in those with intact pancreas.

At present there is little evidence to explain Houssay's results. Shipley and Beyer (1947) suggest that the prolonged anaesthesia with chloralose may be a factor, especially as there is a very high rate of mortality in recently operated alloxan-injected dogs under chloralose.

## SUMMARY.

Guinea-pig pancreas is shown to have as large a store of insulin as that of the rabbit.

Alloxan has no hypoglycemic action when injected subcutaneously or into the blood stream of normal guinea-pigs. Hypoglycemia does occur, however, when alloxan is injected directly into the pancreas of this species.

The absence of hypoglycemia cannot be attributed to an insufficient store of pre-formed insulin. Evidence is given which supports the possibility that the absence of hypoglycemia is due to the relatively high blood reduced-glutathione in guinea-pigs.

Alloxan hypoglycemia does not occur in recently pancreatectomized rabbits.

The conclusion from the above data is that hypoglycemia due to alloxan is pancreatic in origin.

## REFERENCES.

Banerjee, S. (1945): J. biol. Chem., 158, p. 547.
Brückmann, G. and Wertheimer, E. (1947): J. biol. Chem., 168, p. 241.
Goldner, M. G. and Gomori, G. (1944): Endocrinology. 35, p. 241.
Goldner, M. G. and Gomori, G. (1947): Proc. Soc. exp. Biol. Med., 65, p. 18.
Griffiths, M. (1948): J. biol. Chem., 172, p. 853.
Haag, J. R. and Wright, L. D. (1940): J. Nutrition, 19, p. 563.
Houssay, B. A., Orías, O. and Sara, I. (1945): Science, 102, p. 196.
Hughes, H., Ware, L. L. and Young, F. G. (1944): Lancet, 1, p. 148.
Jephcott, C. M. (1932): Trans. Roy. Soc. Canada, 25, p. 183.
Lazarow, A. (1946): Proc. Soc. exp. Biol. Med., 61, p. 441.
Leech, R. S. and Bailey, C. C. (1945): J. biol. Chem., 157, p. 525.
Marks, H. P. and Pak, C. (1936): Bull. Health Org. League of Nations, 5, p. 631.
Marks, H. P. and Young, F. G. (1940): Nature, 146, p. 31.
Richardson, K. C. (1940): Proc. Roy. Soc. London, B, 128, p. 153.
Ridout, J. H., Ham, A. W. and Wrenshall, C. A. (1944): Science, 100, p. 57.
Shipley, E. C. and Beyer, K. H. (1947): Endocrinology, 40, p. 154.
Woodward, G. E. and Fry, E. C. (1932): J. biol. Chem., 97, p. 465.